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13. ABSTRACT *(Maximum 200 Words)*

The overall goal of the proposed study was to provide insights into the molecular mechanisms contributing to the lack of cell-mediated immune response against breast carcinoma (BrCA). Our studies demonstrated that the two known pathways of apoptosis, the death receptor cascade and the mitochondrial cascade, are involved in BrCA-induced T cell death. Over-expression of endogenous inhibitors specific to each of the apoptotic pathways had significant, but partial, reducing effect on the level of the apoptosis induced by interaction with BrCA cells. Therefore, efficient inhibition of T cell death in the tumor microenvironment would require the blocking of the two major apoptotic pathways.

Our studies demonstrated that the anti-apoptotic protein XIAP/hILP undergoes caspase-mediated cleavage in T lymphocytes interacting with BrCA. These observations not only confirm our hypothesis that immune effector cells are induced to apoptose in the tumor microenvironment, but also suggest an intracellular mechanism, which renders T cells susceptible to apoptosis. We identified a clonal Jurkat T cell line that demonstrated a complete resistance to BrCA-induced apoptosis. The block in the apoptotic mitochondrial cascade in this clonal cell line was found to be double-deficiency in Bax and Bak. We identified the molecular mechanism responsible for the deficiency in wild-type Bax (Baxα). The mechanism responsible for the down-regulated expression of Bak remains unknown. Approaches to specifically down-regulate the expression of Bax or Bak in T cells used for adoptive immunotherapy of cancer may protect the T cells from tumor-mediated apoptosis, and thus increase the efficacy of such therapy.

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INTRODUCTION

The overall goal of the proposed study was to provide insights into the molecular mechanisms contributing to the lack of cell-mediated immune response against breast carcinoma (BrCA). We proposed to test the hypothesis that apoptotic cell death is stimulated in T lymphocytes interacting with BrCA cells. This apoptotic cell death can be prevented by treatment with IL-2 or IL-12 cytokines, which upregulate the activity of endogenous protective mechanisms against apoptosis in lymphocytes. The following Technical Objectives were proposed:

1. To investigate the role of death receptors on T cells in BrCA-induced apoptosis of lymphocytes.
2. To identify the intracellular mechanisms of cell death activated in T lymphocytes interacting with BrCA cells.
3. To identify endogenous and cytokine-induced anti-apoptotic mechanisms which can protect lymphocytes from BrCA-induced apoptosis.

We present here our findings regarding the molecular components involved in mediation of, or protection from, death of T lymphocytes in the tumor microenvironment of BrCA patients.

BODY

Section I — Results obtained under Technical Objective 1:

To investigate the role of death receptors on T cells in BrCA-induced apoptosis of lymphocytes

(The major aim of this Objective was achieved. Several of the proposed experiments were not completed because of technical difficulties. For example, we could not separate efficiently TAL from BrCA specimens; also anti-p17 caspase3 Ab were available only in limited quantities and gave high background in immunohistochemistry.)

INVOLVEMENT OF THE DEATH RECEPTORS, FAS AND TRAIL-R, IN BrCA-INDUCED APOPTOSIS OF T LYMPHOCYTES

To examine the ability of BrCA cell lines to induce apoptosis in lymphocytes, BrCA tumor cells were co-cultured with Jurkat T lymphocytes and the loss of DNA in the lymphocytes was assessed by the JAM assay (Fig. 1). Tumor-induced apoptosis of Jurkat cells was dose-dependent (not shown), and first observed following 10h of co-incubation, while death induced by agonistic anti-Fas Ab (CH-11, 200 ng/ml) was detected as early as 2h following the addition of the Ab (not shown). To examine whether the Fas pathway was involved in the observed death of Jurkat cells, anti-FasL neutralizing Ab, anti-Fas neutralizing Ab, or chimeric Fas-Fc construct (capable of blocking FasL) were added to the co-culture of Jurkat and tumor cells (Fig. 1). In the presence of Fas or FasL blocking Ab, as well as Fas-Fc chimeric protein, apoptosis of Jurkat cells induced by BrCA cell lines BT20 or MCF-7 was partially, but significantly inhibited. These results suggest that the Fas-signaling cascade is involved in BrCA-induced apoptosis of interacting Jurkat T cells.

To examine the relevance of Jurkat T cells to tumor-induced apoptosis of T lymphocytes, similar experiments were performed with activated peripheral blood lymphocytes from normal donors (Fig. 2). The experiments were conducted with activated peripheral blood lymphocytes, as resting T cells are not susceptible to death receptor-mediated apoptosis *in vitro* or *in vivo*. Significant level of apoptosis was induced in activated PBL during 16h of co-incubation with either BT20 or MCF-7 BrCA cells. This apoptotic death was partially, but significantly inhibited in the presence of either Fas-Fc or TRAIL-Fc chimeric constructs. These results suggest the both Fas and TRAIL-R are involved in BrCA-induced apoptosis of activated PBL. As the BrCA cell lines BT20 and MCF-7 do not express significant levels of TRAIL, the involvement of TRAIL pathway may relate to activation-induced cell death (AICD) within

the lymphocyte population. In the process of AICD, T-cell stimulation results in expression of surface death ligands, which in turn engage their counter receptors on the same or neighboring cell (1). AICD is mediated mainly by the Fas/FasL death cascade, but the involvement the TRAIL cascade has also been reported (2).

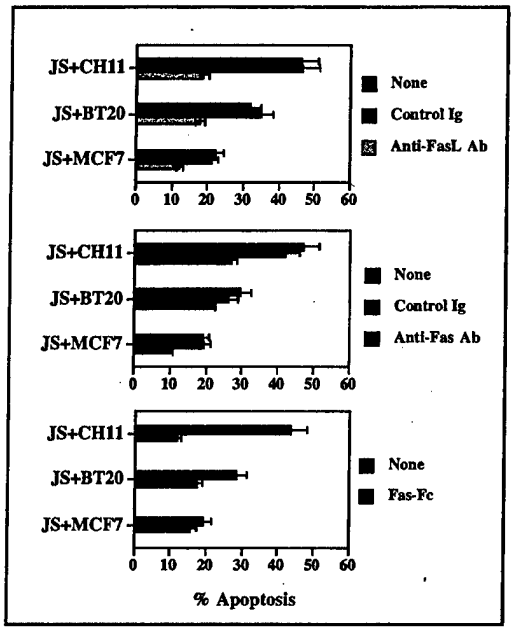


Fig. 1

Fas involvement in BrCA-induced apoptosis of T lymphocytes. BrCA cell lines were co-incubated with [^{3}H]TdR-labeled Jurkat cells at a 40:1 tumor-to-lymphocyte cell ratio for 16 hours. Treatment with agonistic anti-Fas Ab (CH11, 200 ng/ml) served as positive control. Target cell death was determined by measuring fragmentation of ^{3}H-labled target cell DNA. The cultures were incubated in the presence of anti-FasL Ab (4H9, 0.5 μg/ml), antagonist anti-Fas Ab (ZB4, 200 ng/ml), of Fas-Fc fusion protein. The error bars represent the SEM of 8 replicates.

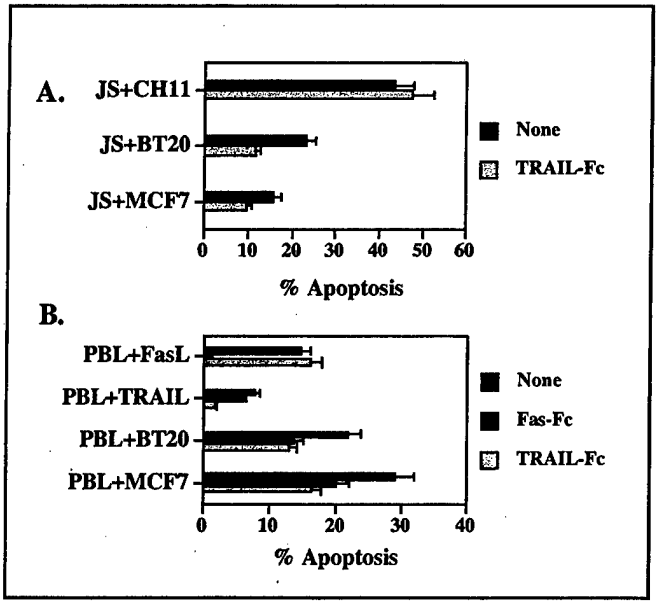


Fig. 2

Fas and TRAIL-R involvement in BrCA-induced apoptosis of activated PBL. In (A), BrCA cell lines were co-incubated with [^{3}H]TdR-labeled Jurkat cells at a 40:1 tumor-to-lymphocyte cell ratio for 16 hours. In (B), BrCA cell lines were co-incubated with [^{3}H]TdR-labeled ConA-activated PBL (for 48 hours) at a 40:1 tumor-to-lymphocyte cell ratio for 16 hours. Treatments with agonistic anti-Fas Ab (CH11, 200 ng/ml) or TRAIL (100 μg/ml) served as positive controls. The cultures were incubated in the presence or absence of Fas-Fc or TRAIL-Fc fusion proteins. The error bars represent the SEM of 8 replicates.

Summary and conclusions (Section I)

In this part of the study we demonstrated that BrCA-induced apoptosis of interacting T lymphocytes is mediated by the extrinsic/death receptor apoptotic cascade. This cascade is initiated either by a mechanism of AICD via ligands expressed on activated lymphocytes (FasL, TRAIL) or directly by BrCA cells that express FasL.

These findings will be included in a manuscript on mechanisms involved in BrCA-induced apoptosis of T lymphocytes that we currently prepare.

Section II — Results obtained under Technical Objective 2:

To identify the intracellular mechanisms of cell death activated in T lymphocytes interacting with BrCA cells

(The major part of Technical Objective 2 has been achieved. We did not follow a small part of the proposed experiments, because they lost some of the initial rationale as the study progressed.)

II.1 A ROLE FOR CASPASES IN BrCA-INDUCED APOPTOSIS OF T LYMPHOCYTES

To investigate the role of caspases in tumor-induced apoptosis of T cells, Jurkat target cells were pretreated with the irreversible peptide inhibitors Z-VAD-FMK or Z-IETD-FMK. BrCA-induced apoptosis of Jurkat cells was significantly inhibited by the pan-caspase inhibitor Z-VAD-FMK, and also by the caspase-8 inhibitor, Z-IETD-FMK.

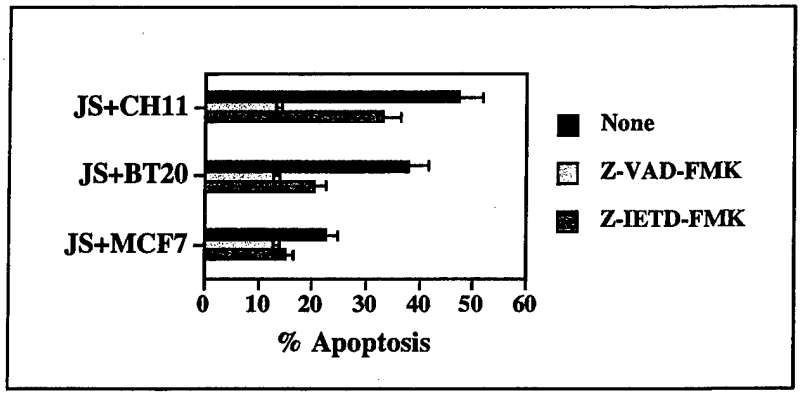


Fig. 3

BrCA-induced apoptosis of T lymphocytes is mediated by caspases. [^{3}H]TdR-labeled Jurkat cells were pretreated with Z-VAD-FMK or Z-IETD-FMK (100 μM each) for 2 hours, and then co-incubated with BrCA cell lines at a 40:1 tumor-to-lymphocyte cell ratio for additional 16 hours. T cell apoptosis was determined by measuring the loss of ^{3}H-labeled target cell DNA. The error bars represent the SEM of 8 replicates.

II.2 A ROLE FOR MITOCHONDRIA IN BrCA-INDUCED APOPTOSIS OF T LYMPHOCYTES

To elucidate the significance of the mitochondria in BrCA-induced T-cell death, we investigated the effects of various inhibitors of mitochondrial pathways. Reactive Oxygen Species or oxidants (ROS) are formed in the mitochondria, but become toxic when present in excessive amounts, causing oxidative damage. To assess the significance of a redox imbalance, we used the antioxidants, diphenyleneiodonium chloride (DPI, 25 μM), a specific inhibitor of flavin-dependent oxidoreductase, and pyrrolidene-dithiocarbamate (PDTC), a radical scavenger (3, 4). Prior to incubation with tumor cells or agonistic anti-Fas Ab, Jurkat cells were treated with these antioxidants for 2h. The effects of these inhibitors on tumor-induced apoptosis of lymphocytes were assessed by the JAM assay. As shown in Figure 4A, loss in ^{3}H-labeled DNA in Jurkat cells co-incubated with BrCA cells was significantly reduced in the presence of either one of these antioxidants. These results suggest that tumor-induced apoptosis of T cells involves the generation of ROS and is significantly inhibited by specific antioxidants.

Next, the effects of two mitochondria-specific inhibitors, bongkrekic acid (BA) and cyclosporine A (CsA) were examined. BA, a specific inhibitor of permeability transition and a ligand of adenine nucleotide translocator (ANT) in the inner mitochondrial membrane, can inhibit the pre-apoptotic $\Delta\Psi_m$ disruption (5, 6). CsA prevents mitochondrial permeability transition by blocking translocation of mitochondria matrix-specific cyclophilin–D to the mitochondria inner membrane, thereby decreasing the sensitivity of mitochondrial megachannels to calcium ions. As shown in Figure 4B, BA significantly ($p<0.01$, Mann-Whitney *U*) blocked mitochondria-dependent apoptosis of Jurkat cells induced by etoposide, but had no effect on death induced by agonistic anti-Fas Ab tested at various concentrations.

However, a significant inhibitory effect of BA was detected on the level of tumor-induced apoptosis. CsA had no inhibitory effect on Jurkat cell apoptosis induced by either anti-Fas Ab, VP16, or BrCA tumor cells. These results further indicate that tumor-induced apoptosis of T cells has a component which is mitochondria-dependent, in contrast to the apoptotic cascade induced by direct ligation of surface Fas.

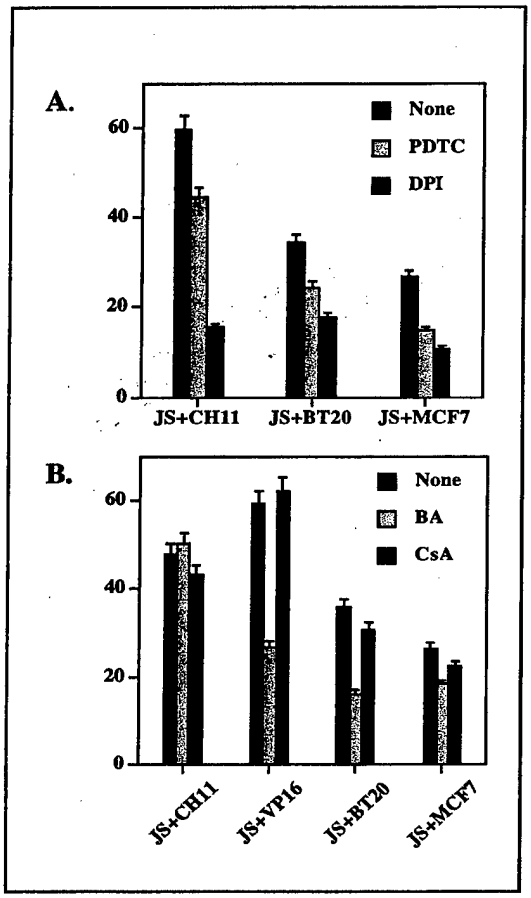


Fig. 4

Inhibitors of the mitochondrial apoptotic pathway partly block BrCA-induced apoptosis of T lymphocytes. In (A), [^{3}H]TdR-labeled Jurkat cells were pretreated with the antioxidants DPI or PDTC (25 μM each) for 2 hours. In (B), [^{3}H]TdR-labeled Jurkat cells were pretreated with the permeability transition inhibitors, bongkrekic acid (BA, 50 μM) or cyclosporin A (CsA, 25 μM) for 2 hours. After co-incubation with BrCA tumor cells, loss of ^{3}H-labeled target cell DNA was measured. Treatments with anti-Fas Ab (CH11, 200 ng/ml) or VP16 (20 μM) for 16 hours served as positive controls for death receptor- or mitochondrial-mediated apoptosis, respectively. The error bars represent the SEM of 8 replicates.

Summary and conclusions (Section II.1 and II.2)

In this part of the study, we demonstrated that mitochondria are involved in the apoptotic cascades induced in T cells by either Fas ligation or tumor cells. However, these two cascades are differentially susceptible to a panel of inhibitors of mitochondrial apoptotic events. Whereas Fas-mediated apoptosis in Jurkat cells is executed in the presence of mitochondria specific inhibitors, tumor-induced apoptosis is partially inhibited, suggesting that it is significantly amplified by a mitochondrial cascade.

A part of these findings has been published in the following report:
Gastman, B. R., Yin, X. M., Johnson, D. E., Wieckowski, E., Wang, G. Q., Watkins, S. C., and Rabinowich, H. Tumor-induced apoptosis of T cells: amplification by a mitochondrial cascade. Cancer Res. 60: 6811-6817, 2000.

The results on mitochondria involvement in BrCA-induced apoptosis will be included in a manuscript we currently prepare for publication.

II.3. ASSESSMENT OF PROTECTIVE MECHANISMS AGAINST BrCA-INDUCED APOPTOSIS OF T LYMPHOCYTES

The studies described below were proposed as a part of Technical Objectives 2 and 3.

Results

The results presented in Sections I and II of this report suggested that the two major pathways of apoptosis, the Fas death receptor and the mitochondrial pathway are actively involved in tumor-induced apoptosis of T cells. To further investigate the contribution of the two apoptotic pathways, we examined the efficiency of various inhibitors of apoptosis in protecting T cells from tumor-induced apoptosis. To this end, variants of Jurkat cells resistant to either death receptor or mitochondria apoptotic pathways,

were co-incubated with tumor cells, and their susceptibility was assessed by measuring a loss in DNA as determined by the JAM assay. Stably transfected Jurkat cells with either CrmA, FLIP-L or FLIP-S, which abrogated completely the response to agonistic anti-Fas Ab (7-9), remained susceptible to death induced by tumor cells (Fig. 5). Also, when mitochondrial apoptotic pathway was blocked by stable transfection of Bcl-X_L (10), these Jurkat T cells were partially susceptible to tumor-induced apoptosis, which could potentially proceed via a death receptor pathway. Full resistance to tumor-induced apoptosis was observed in a clonal variant of Jurkat T cells, in which both the Fas and the mitochondrial pathways of apoptosis were completely arrested (Fig. 6). These experiments provided an additional proof for the activation of both the mitochondrial and the death receptor apoptotic cascades in T cells interacting with BrCA. The resistance of this clonal Jurkat cell line to the Fas and the mitochondrial cascades induced by anti-Fas Ab or VP-16, respectively, is shown in Figure 6B.

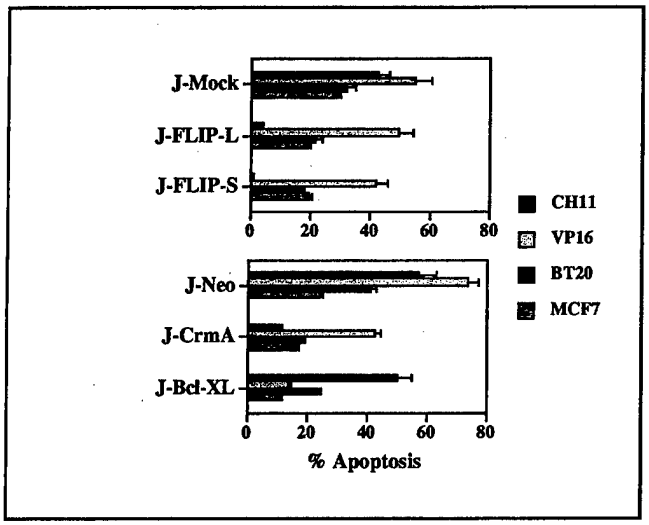


Fig. 5

Endogenous inhibitors of the extrinsic pathway (FLIP-L, FLIP-S, CrmA) and of the intrinsic pathway (Bcl-XL) partially block BrCA-induced apoptosis of T lymphocytes. [^{3}H]TdR-labeled Jurkat cells stably transfected with FLIP-L or FLIP-S (A), or CrmA or Bcl-XL (B) were treated for 16 hours with CH11 or VP16, or co-incubated with BrCA cell lines at 40:1 tumor-to-lymphocyte cell ratio. Apoptosis was measured loss of ^{3}H-labeled DNA. The error bars represent the SEM of 8 replicates.

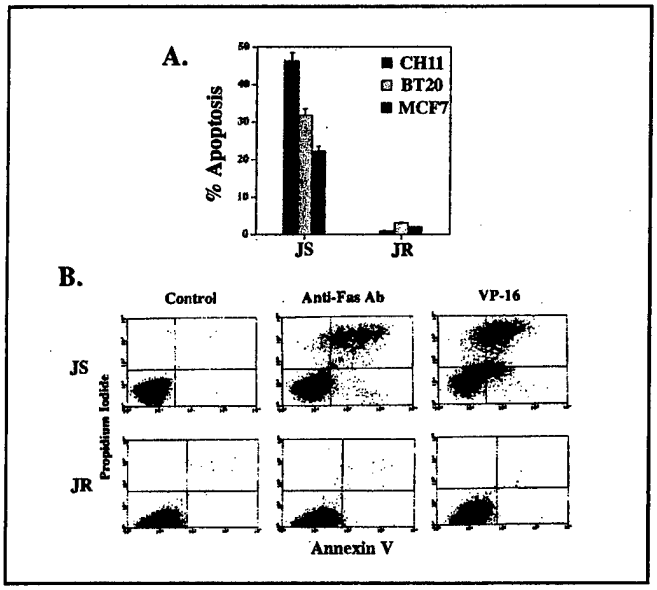


Fig. 6

Complete inhibition of BrCA-induced apoptosis in a clonal Jurkat cell line resistant to both Fas- and mitochondrial apoptotic pathways. In A, resistance of Jurkat cells to BrCA-induced apoptosis was assessed by loss in [^{3}H]TdR-labeled DNA. [^{3}H]TdR-labeled clonal Jurkat cell lines were co-incubated with BT20 or MCF7 BrCA cells for 16 hours. The error bars represent the SEM of 8 replicates. In B, the resistance to Fas and VP-16 apoptotic signals was assessed by flow cytometry. Clonal Jurkat cell lines were treated with agonistic anti-Fas Ab or VP-16. The cells were then stained with Annexin V (2 μg/ml) and propidium iodide (5 μg/ml), and the presence of apoptotic cells was assessed by flow cytometry.

Summary and conclusions (Section II.3)

Significant, but partial, inhibition of tumor-induced apoptosis of T lymphocytes was mediated by endogenous inhibitors of either the death receptor pathway or of the mitochondrial cascade. However, complete inhibition of tumor-induced cell death was observed only in lymphocytes where both pathways were blocked.

Section III — Results obtained under Technical Objective 3:

To identify endogenous and cytokine-induced anti-apoptotic mechanisms which can protect lymphocytes from BrCA-induced apoptosis.

(Most of our findings were obtained by the experiments proposed in the various tasks of Technical Objective 3. Understandably, the experimental design followed the results. We successfully analyzed molecular mechanisms underlying the resistance of T cells to apoptosis. To simplify the experimental approach, we used apoptotic agents known to induce mitochondrial cascade in a similar fashion to that of BrCA. We currently analyze the protective mechanisms induced by cytokines. This part of the study is not completed, as we dedicated most of our efforts to the analysis of endogenous anti-apoptotic mechanisms.)

III.1. THE FATE OF ENDOGENOUS PROTEIN INHIBITORS OF APOPTOSIS IN T LYMPHOCYTES IN THE TUMOR MICROENVIRONMENT

The two pathways of apoptosis are regulated by either specific or shared endogenous inhibitors of apoptosis. Thus, cellular FLIP, which serves as a dominant negative caspase-8, blocks caspase-8 activity in the initiation phase of Fas or TRAIL death receptor pathways (11, 12). Bcl-2 and Bcl-X_L block preferentially mitochondrial apoptotic cascade (13). The two apoptotic pathways converge on caspase-3, which is efficiently blocked by the endogenous protein XIAP/hILP (X-linked inhibitor of apoptosis/human IAP-like protein) (14). The levels of expression and proper function of endogenous inhibitors of apoptosis appear to be significant determining factors for cell survival. In preliminary studies, we observed reduced levels of expression of Bcl-2, Bcl-X_L and XIAP/hILP in T cells interacting with tumor cells. We investigated the mechanisms responsible for the observed down-regulation in XIAP/hILP protein expression.

A part of these findings has been published in the following report:
Johnson, D. E., Gastman, B. R., Wieckowski, E., Wang, G. Q., Amoscato, A., Delach, S. M., and Rabinowich, H. Inhibitor of apoptosis protein hILP undergoes caspase-mediated cleavage during T lymphocyte apoptosis. Cancer Res. 60: 1818-1823, 2000.

ALTERED EXPRESSION OF XIAP/hILP IN T LYMPHOCYTES INTERACTING WITH TUMOR CELLS

Background

The execution of cellular apoptosis involves the activation of a cascade of intracellular proteases belonging to the caspase protease family (15, 16). Caspases are initially synthesized as inactive proenzymes, and activation involves processing to smaller active subunits. Activation of the apical proteases, caspase-8 (17-20), following engagement of cell surface death receptors, or caspase-9 (8, 9), following release of cytochrome c from mitochondria, results in processing and activation of downstream executioner caspases including caspase-3 (21, 22). Executioner caspases cleave specific cellular substrate proteins, facilitating the demise of the cell (15, 16).

A number of intracellular proteins that negatively regulate apoptosis execution, primarily by interfering with the caspase cascade, have been identified. Antiapoptotic members of the Bcl-2 protein family act to prevent release of cytochrome c from the mitochondria (23, 24), and also can bind and incapacitate Apaf-1 (25, 26) a critical cytoplasmic protein involved in cytochrome c-mediated activation of caspase-9 (21, 27). c-FLIP, a death-effector domain-containing protein, prevents association of caspase-8 with cell surface death receptors, thereby blocking caspase-8 activation (9). More recently, it has been shown that members of the IAP (14) protein family bind and inhibit specific caspases (28-30).

Human IAP proteins, including hILP/XIAP (31, 32), c-IAP1, c-IAP2, NAIP, Survivin, and Bruce, are characterized by the presence of one to three copies of a seventy-amino acid motif, the BIR domain, which bears homology to sequences found in the baculovirus IAP proteins (reviewed in 17). The hILP, c-IAP1, and c-IAP2 proteins also contain carboxyl-terminal RING finger domains. hILP, c-IAP1, and c-IAP2 bind and inhibit the activated, but not the proenzyme, forms of caspase-3 and –7 (14, 28, 30). In addition, hILP, c-IAP1, and c-IAP2 bind procaspase-9, preventing processing and activation of this enzyme. These inhibitors also inhibit active caspase-9. However, despite demonstrations that hILP, c-IAP1, and c-IAP2 can bind and inhibit caspases, the molecular mechanism(s) of this inhibition remains unclear. Two caspase inhibitor proteins that are unrelated to IAPs, cowpox viral CrmA (8) and baculovirus p35 (7, 33), also bind directly to caspases (7). CrmA and p35 have been shown to be suicide inactivators of caspases (34-36). Following binding, CrmA and p35 are proteolytically cleaved, and the cleaved products remain associated with the caspase to inhibit enzyme activity.

The hILP protein consists of three BIR domains and one RING finger domain, and appears to be a more potent inhibitor of caspase-3 and -7 than c-IAP1 or c-IAP2 (29, 30). The RING finger domain is not essential for hILP binding to caspase-3 and -7, but is important for binding to the cytoplasmic domain of bone morphogenetic protein type I receptor and may mediate functions of hILP that are unrelated to apoptosis (37). Of the three hILP BIR domains, the second BIR domain, but not the first or third domains, is sufficient for binding and inhibition of caspase-3 and -7 (38). Thus, only a portion of the molecule is needed for caspase inhibition and suppression of apoptosis.

Results

Cleavage of hILP in apoptotic Jurkat cells. To determine the fate of cellular hILP protein during apoptotic execution, Jurkat T leukemic cells were stimulated with 200 ng/ml agonistic anti-Fas Ab for varying lengths of time. Following stimulation, whole cell lysates were prepared and analyzed by immunoblotting using mAb raised against amino acids 268-426 of the human hILP protein. As expected, full-length hILP was detected as a 57 kDa protein (Fig. 7A). Following 16 hours of stimulation with anti-Fas Ab, the level of full-length hILP was significantly reduced. Even more apparent was the appearance of a 29-kDa fragment recognized by the anti-hILP mAb. The 29-kDa fragment was first detected after 2 hours of stimulation, and its levels continued to increase thereafter. Stimulation of Jurkat cells with anti-Fas Ab also resulted in the processing of procaspase-3 (32 kDa) to active subunits of 20 and 17 kDa (Fig. 7B). Activation of caspase-3 was first detected after 2 hours, but was far more substantial after 8 hours of treatment. Thus, cleavage of hILP occurred concurrently with caspase-3 activation.

Cleavage of hILP to a 29-kDa fragment was also observed following treatment with 20 μM VP-16 or 0.5 μM staurosporin (Fig. 7A). The 29-kDa fragment was first detected after 4 hours of treatment with VP-16 and after 2 hours of treatment with staurosporin. Processing of caspase-3 coincided with production of the hILP fragment. The 29-kDa fragment of hILP was also detected in Jurkat cells

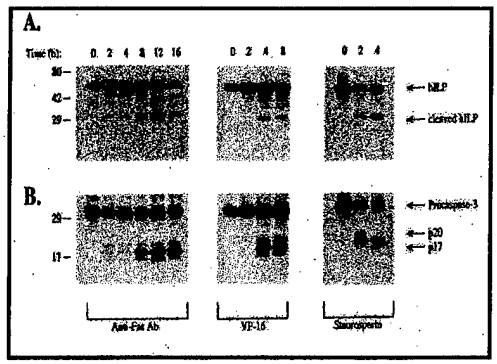


Fig. 7. Cleavage of hILP protein during apoptosis induced by anti-Fas Ab, VP-16 or staurosporin. Jurkat T cells were treated with agonistic anti-Fas mAb, VP-16 or staurosporin at 37°C for varying lengths of time. Following treatment, whole cell lysates were prepared. Proteins detected by immunoblotting. In A, the membranes were probed with anti-hILP mAb. In B, the membranes were stripped and reprobed with polyclonal anti-caspase-3 Ab.

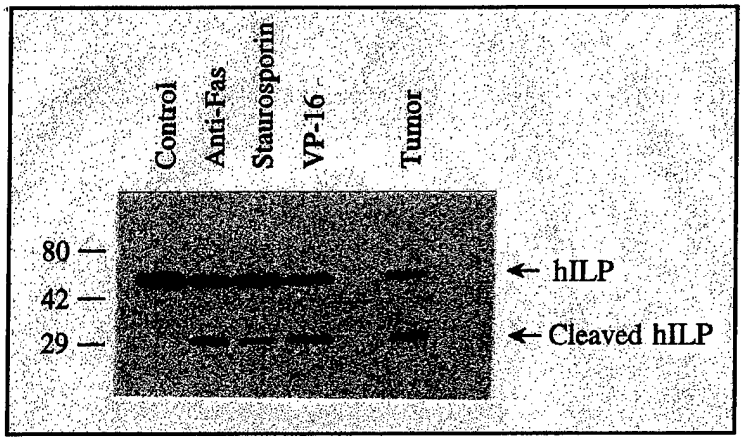


Fig. 8
Cleavage of hILP in Jurkat cells co-incubated with BrCA cells. Jurkat cells were incubated with BrCA cells for 16h at a tumor-to-lymphoyte cell ratio of 20:1. Jurkat cells were then negatively selected by removal of tumor cells with the use of anti-$\alpha_6\beta_4$ mAb and magnetic beads. Negatively selected Jurkat cells were lysed and analyzed by Westren blotting for expression of hILP.

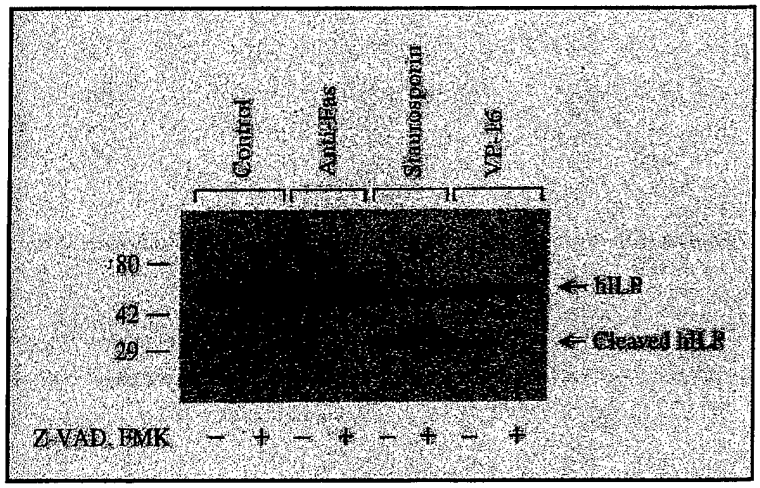


Fig. 9
Inhibition of hILP cleavage by the caspase inhibitor Z-VAD-FMK. Jurkat cells were treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM, or VP-16 (20 μM) in the presence or absence of Z-VAD-FMK (50 μM). Whole cell lysates were electrophoresed on 15% SDS gels and analyzed by immunoblotting using anti-hILP mAb.

Inhibition of hILP cleavage by Z-VAD-FMK. A number of intracellular proteins are cleaved by caspase proteases during apoptosis, including the antiapoptotic molecules Bcl-2 and Bcl-X_L (39-42). In addition, the CrmA and p35 proteins, which directly bind and inhibit caspases, have been shown to be caspase substrates (34-36). Since hILP is known to bind and inhibit caspase-3 and -7, we sought to determine whether hILP cleavage in apoptotic cells was mediated by a caspase protease. To address this question, Jurkat cells were treated with anti-Fas, staurosporin, or VP-16 in the absence or presence of Z-VAD-FMK, a potent, general inhibitor of caspases. As shown in Figure 9, Z-VAD-FMK completely abrogated production of the 29-kDa-hILP fragment in response to all three stimuli. This indicated that hILP cleavage was caspase mediated.

Inhibition of hILP cleavage by Bcl-2 and CrmA. To further examine the involvement of caspases in hILP cleavage we studied the effects of two inhibitors of caspase activation, Bcl-2 and CrmA. Bcl-2 inhibits caspase activation by blocking release of cytochrome c from the mitochondria, and also may interact with Apaf-1, disrupting caspase-9 activation (23-26). Because Bcl-2 blocks cytochrome c release, it is a potent inhibitor of stimuli that primarily utilize the mitochondrial pathway of apoptosis, such as chemotherapeutic agents. By contrast, Bcl-2 is much less efficient at inhibiting Fas-mediated apoptosis, which is not dependent on the mitochondrial pathway. The CrmA protein binds and potently inhibits caspase-8, the apical caspase in Fas-mediated signaling (7). Thus, CrmA strongly inhibits anti-Fas-induced apoptosis. On the other hand, chemotherapy-induced caspase activation and

apoptosis is only modestly inhibited by CrmA. Jurkat cells engineered to overexpress Bcl-2 or CrmA (43) were treated with anti-Fas antibody, staurosporin, or VP-16, followed by immunoblot analysis with anti-hILP (Fig. 10). As a control, cells transfected with vector alone (Neo) were also analyzed. Bcl-2 overexpression completely abrogated hILP cleavage in response to VP-16 treatment. Considerably less protection was seen in Bcl-2/Jurkat cells treated with anti-Fas or staurosporin. CrmA, on the other hand, dramatically inhibited hILP cleavage in the anti-Fas- and staurosporin-treated cells, but had little impact in VP-16-treated cells. Taken together, these results support the conclusion that cleavage of hILP in apoptotic cells is mediated by a caspase protease.

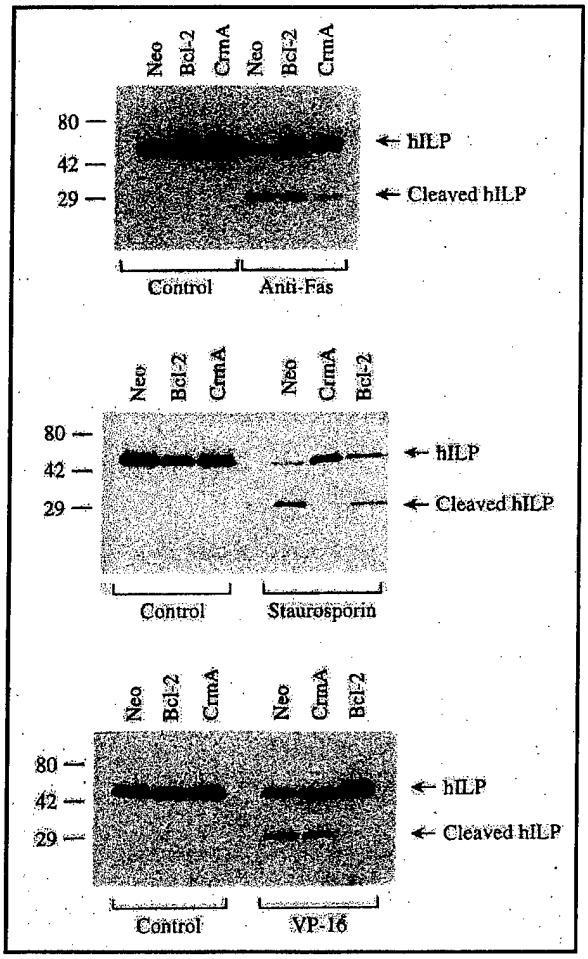


Fig. 10

Inhibition of hILP cleavage by Bcl-2 and CrmA. Clonal Jurkat cell lines engineered to overexpress Bcl-2 or CrmA were treated as described in Fig. 8. Jurkat cells transfected with vector alone (Neo) served as control. Following treatment, whole cell extracts were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti-hILP mAb.

Cleavage of hILP by recombinant caspases. To identify caspases involved in hILP cleavage, *in vitro* translated ^{35}S-hILP protein was prepared and incubated with active, recombinant caspase-3 or caspase-7. As assessed by autoradiography, a p29 fragment was generated by each of the two caspases (Fig. 11A and B). Production of the cleavage fragments was inhibited by the caspase inhibitor Z-DEVD-FMK. It appears that *in vitro* translated hILP is more accessible to recombinant caspases than the endogenous hILP is to endogenous caspases, as additional proteolytic fragments were detected *in vitro*. However, like the endogenous fragment, the p29 cleavage fragment generated from *in vitro* translated hILP was detected by anti-hILP mAb (Fig. 11C and D).

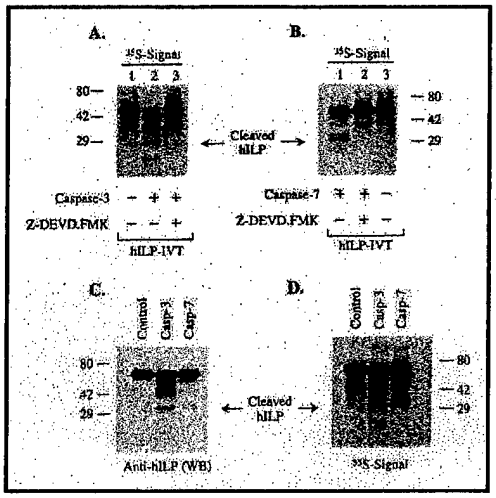


Fig. 11

In vitro translated ^{35}S-hILP is cleaved by recombinant caspase-3 or caspase-7. In vitro translated ^{35}S-hILP was treated for 1h with recombinant caspase-3 (A) or caspase-7 (B, 0.2 μg of enzyme per reaction) in the presence or absence of Z-DEVD-FMK (50 μM). The reaction products were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were analyzed autoradiography (A, B, and D) or by Western blotting with anti-hILP mAb (C).

As hILP has been reported to bind caspase-3 and caspase-7 (30, 38), we examined whether the p29 fragment maintains its ability to physically associate with caspases. To this end, *in vitro* translated ^{35}S-hILP was treated with recombinant caspase-3 or caspase-7. The caspases were then immunoprecipitated by specific anti-caspase Ab, and resolved on SDS gels. The ^{35}S-labeled co-immunoprecipitated proteins were examined by autoradiography. As shown in Figure 12, the p29 fragment was not only immunoprecipitated by anti-hILP Ab, but also co-immunoprecipitated with either caspase-3 or caspase-7. These results demonstrate that although hILP is cleaved by caspases, the p29 product remains associated with the cleaving enzyme. Thus, hILP may function in a similar fashion to CrmA and p35, with the cleaved products remaining bound to the enzyme and inhibiting the caspase activity. Alternatively, hILP may behave like Bcl-2, losing its anti-apoptotic activity following cleavage, and perhaps even becoming proapoptotic (41).

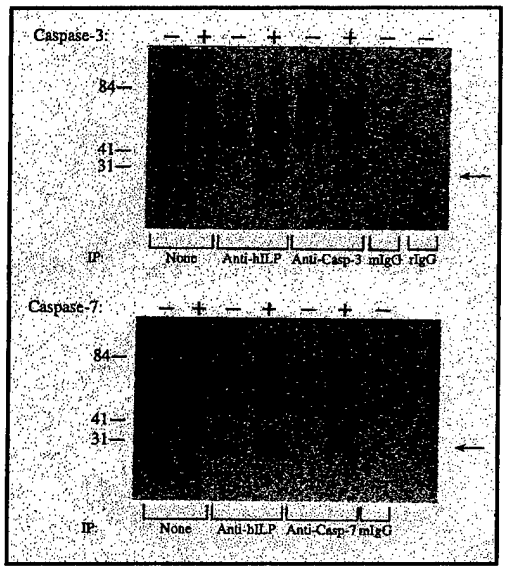


Fig. 12

Association of the p29 cleavage product of ^{35}S-hILP with recombinant active subunits of caspase-3 or caspase-7. In vitro translated ^{35}S-hILP was treated for 1h at 30°C with recombinant caspase-3 (Top) or recombinant caspase-7 (Bottom). Following treatment, immunoprecipitating Abs, specific for hILP, caspase-3 or caspase-7 were added. Immune-complexes were selected using either Protein G (for anti-hILP or anti-caspase-7 mAb) or Protein A (for anti-caspase-3 Ab). The immune-complexes were resolved on 15% SDS gels, transferred to PVDF membranes, and examined by autoradiography. As controls, the in vitro translated ^{35}S-hILP was subjected to no immunoprecipitation (none) or immunoprecipitated with anti-hILP, mouse IgG or rabbit IgG. The arrow indicates the p29 cleavage product immunoprecipitated by anti-hILP, or co-immunoprecipitated with either anti-caspase-3 or anti-caspase-7.

Mapping of the hILP cleavage site. To identify the site of cleavage responsible for generating the 29-kDa product, we prepared constructs in which aspartic acid-59 296, 309 or 242 in hILP was replaced by alanine. The mutated recombinant proteins were treated with recombinant caspase-3 and assessed by immunoblotting for the presence of a 29-kDa cleavage product. All mutated proteins tested underwent cleavage similar to that of wild type, whereas hILP D242A was not cleaved (Fig. 13).

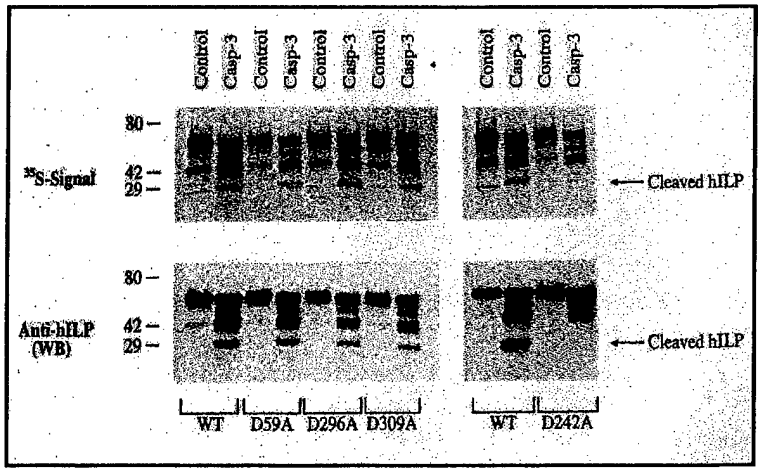


Fig. 13

hILP is cleaved at aspartic acid-242. cDNA encoding full length hILP or hILP mutants as indicated were translated in vitro in reticulocytes lysate containing ^{35}S-methionine. The ^{35}S-radiolabeled hILP proteins were incubated with 100 nM caspase-3 for 1 hr at 37°C. Reaction products were then resolved on SDS gels and analyzed by autoradiography.

Cleavage of hILP in T-cell receptor-mediated apoptosis. To investigate the relevance of hILP cleavage in normal T lymphocytes, peripheral blood T cells from healthy individuals were stimulated to undergo AICD by incubation with immobilized anti-CD3 mAb in the presence of PMA and ionomycin for 14h at 37°C (44). As assessed by Western blot analyses performed on whole cell extracts, the p29 fragment was detected in T cells activated via the TcR (Fig. 14A, Lane 1), but not in control cells stimulated with only PMA and ionomycin (Fig. 14A, Lane 4). Interestingly, in human peripheral blood T cells, an additional p45 hILP protein was detected in cells treated with anti-CD3, VP-16 or staurosporin, suggesting that hILP may be subjected to cleavage prior to production of the p29 fragment. In T lymphocytes pretreated with the pan-caspase inhibitor Z-VAD-FMK prior to stimulation of AICD no cleavage products of hILP were detected (data not shown).

Detection of p29 hILP in OvCA ascitic TAL. To further investigate the physiologic significance of hILP cleavage, we examined T cells purified from either OvCA ascitic TAL or T cells from peripheral blood of normal donors. OvCA TAL were used in these experiments as they can be obtained in substantial numbers when separated from ovarian ascites. The OvCA TAL population contained a substantial percentage of apoptotic cells (up to 30% TUNEL-positive T cells) (45). Whole cell extracts were prepared from TAL-T or PBL-T, and examined by Western blot analysis for the presence of cleaved products of hILP. The p29 cleaved product of hILP was detected in TAL-T prepared from ascites of 4 OvCA patients, but not in PBL-T from two normal donors (Fig. 14B). These results demonstrate that the cleavage of hILP occurs in an *in vivo* setting where the loss or gain of inhibitory function may have significant biological consequences.

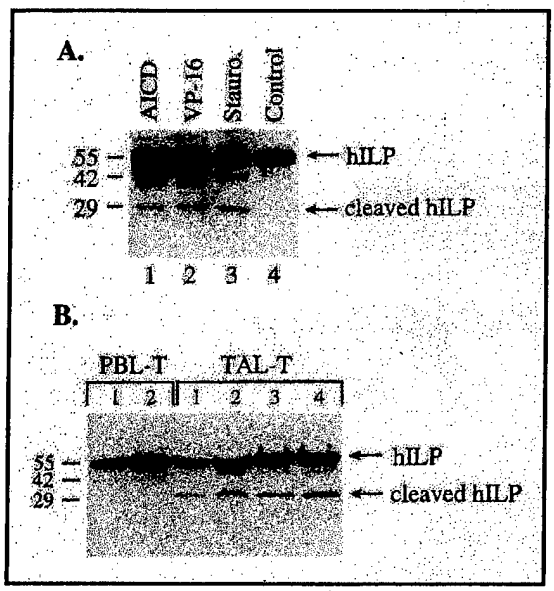


Fig. 14

Detection of the p29 fragment of hILP in PBL-T cells stimulated to undergo AICD (A) and in OvCA ascitic TAL-T cells (B). In A, peripheral blood T cells from a normal donor were stimulated by immobilized anti-CD3 mAb (5 μg/ml), PMA (50 ng/ml) and ionomycin (0.5 μg/ml) to induce AICD (lane 1). PBL-T stimulated by PMA and ionomycin served as negative control (lane 4), and cells stimulated with VP-16 (lane 2, 20 μM) or staurosporin (lane 3, 0.5 μM) served as positive controls. Similar results were obtained with PBL-T cells from 3 normal individuals. In B, TAL-T cells from four OvCA ascites (1-4) and control PBL-T from two normal donors (1-2) were purified by negative selection as described in Material and Methods. Cell lysates were resolved by 15% SDS-PAGE, transferred to PVDF membranes, and probed with hILP-specific mAb.

In summary, we have shown that endogenous hILP is cleaved by a caspase protease during cellular apoptosis. The fact that hILP cleavage is seen following Fas stimulation or treatment with VP-16 or staurosporin indicates that the caspase responsible is active in both death receptor- and drug-mediated apoptotic pathways. The observation that the hILP cleavage products remain associated with active subunits of caspase-3 and –7, suggests that hILP, like CrmA and p35, may act as a suicide inactivation of caspases, undergoing cleavage as a part of its mechanism. However, it is also possible that the cleavage products exhibit some unique cellular function. In this regard, it is interesting to note that sequences in the RING finger domain of hILP promote association of hILP with the cytoplasmic domain

of bone morphogenetic protein type I receptor (37). If the hILP cleavage products dissociate from caspases inside the cell, then it is likely that they may act as dominant-negative inhibitors of normal hILP function. Future studies will be needed to thoroughly investigate these possibilities. Our findings that hILP cleavage products are found in peripheral blood T cells undergoing AICD and also in TAL demonstrate that hILP proteolysis occurs *in vivo*. Thus, hILP cleavage may be fundamentally important to the process of apoptosis in both normal and pathologic *in vivo* settings.

Summary and conclusions (Section III.1)

In studying the expression of hILP in apoptotic T cells, we observed that this protein was cleaved, generating at least one prominent fragment of 29-kDa. This p29 fragment was detected in T lymphocytes treated with agonistic anti-Fas Ab, VP-16 or staurosporin. It was also expressed in tumor-associated lymphocytes (TAL) in T cells stimulated to undergo AICD, and in T cells co-incubated with BrCA cells. Cleavage of hILP was also observed in cell-free reactions using *in vitro* translated hILP and recombinant caspase-3 or -7. Utilizing site-directed mutagenesis, we mapped the cleavage site responsible for generating p29 to Aspartic acid 242. Our results suggest that loss in expression of hILP is a molecular mechanism involved in susceptibility of T lymphocytes to apoptosis mediated by BrCA.

III.2. IDENTIFICATION OF MOLECULAR MECHANISMS OF RESISTANCE TO APOPTOSIS IN T LYMPHOCYTES

To identify endogenous mechanisms of resistance to apoptosis in T lymphocytes that might be relevant to death induced by BrCA, we investigated the mechanisms involved in the complete resistance of the variant of Jurkat cells we identified. Our initial efforts were directed at the cellular characterization of the observed resistance, and later on, at investigating the molecular mechanisms underlying this resistance. Parts of these studies were published in the following manuscripts:

1. Wang, G. Q., Gastman, B. R., Wieckowski, E., Goldstein, L. A., Rabinovitz, A., Yin, X. M., and Rabinowich, H. Apoptosis-resistant mitochondria in T cells selected for resistance to Fas signaling. J Biol Chem. 276: 3610-3619, 2001.

2. Wang, G. Q., Gastman, B. R., Wieckowski, E., Goldstein, L. A., Gambotto, A., Kim, T. H., Fang, B., Rabinovitz, A., Yin, X. M., and Rabinowich, H. A role for mitochondrial Bak in apoptotic response to anticancer drugs. J Biol Chem. 276: 34307-34317, 2001.

III.2.1. CELLULAR CHARACTERIZATION OF A T CELL VARIANT RESISTANT TO TUMOR-INDUCED APOPTOSIS

The clonal Jurkat cell line that was completely resistant to tumor-induced apoptosis was selected for resistance to Fas-induced apoptosis and was found to be cross-resistant to VP-16 or staurosporin (Fig. 6). Each of the apoptotic pathways was blocked at an apical phase, where common regulators of apoptosis have not yet been defined. The Fas pathway was blocked at the level of caspase-8, whereas the intrinsic pathway was blocked at the mitochondria. Wild-type Jurkat sensitive (J-S) and Jurkat resistant (J-R) cell lines expressed similar protein level of caspase-8 (Fig. 15). However, in contrast to caspase-8 in J-S cells, caspase-8 in J-R cells was not processed into its active subunits upon stimulation with agonistic anti-Fas Ab (Fig. 15). Thus, apoptotic events downstream of caspase-8 did not occur, including caspase-3 processing, or the cleavages of the TCR-ζ protein, Bcl-2, BCL-XL, PARP, or XIAP/hILP (Figs. 15 and 16).

We determined that the block in the Fas-pathway was at the level of caspase-8, as there was no difference in the levels of expression of Fas or FADD required for the Fas-signaling. In addition, the ligation of each of two death receptors known to be dependent on caspase-8, Fas and TRAIL-Rs, did not lead to caspase-8 processing in J-R cells. These latter observations suggested that the block in signaling was at the caspase-8 level shared by both receptors, and not upstream of caspase-8. To determine potential differences between caspase-8 in the two cell lines we performed a comprehensive gene analysis. By DNA sequencing of either J-S or J-R clones we identified known and also unknown isoforms of caspase-8, but we did not detect differences that could explain the lack of activation of caspase-8 in the resistant cells. In particular, we did not detect differences in either the cleavage or the catalytic sites of paired caspase-8 isoforms from J-S or J-R cells.

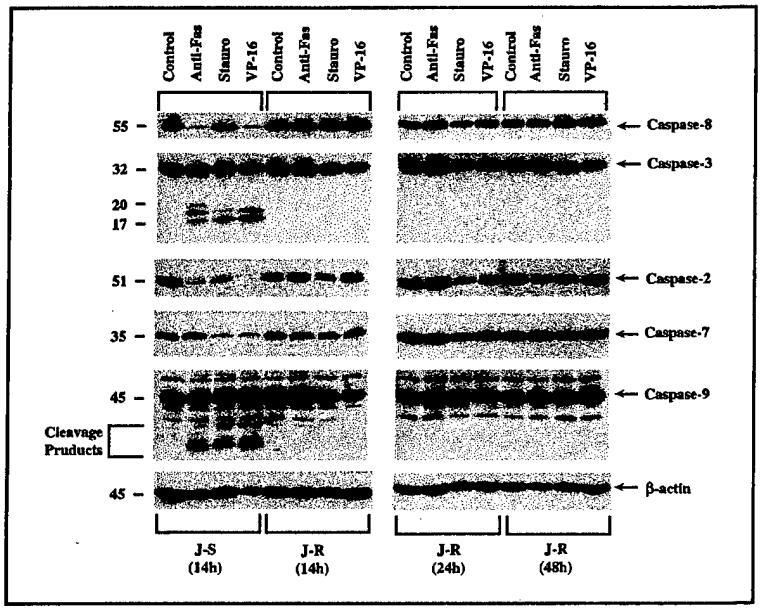


Fig. 15

Abrogation of caspase processing in resistant clonal Jurkat cells. Wild-type and resistant Jurkat cells were treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) for 14, 24 or 48 hr. At the end of the treatment period, whole cell extracts were separated by SDS-polyacrylamide gels, and resolved proteins were transferred to a PVDF membrane. The processing of the indicated caspases was assessed by immunoblotting with specific Abs as detailed in Materials and Methods. Equal amounts of protein were loaded after quantification, and immunoblotting for β-actin served as an additional loading control. Each panel represents the results of at least three experiments.

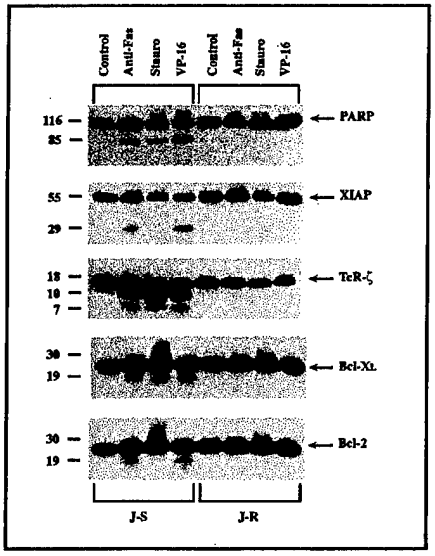


Fig. 16

Abrogation of caspase cleaving activity of endogenous substrates in resistant clonal Jurkat cells. Wild-type and resistant Jurkat cells were treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) for 14 hr. At the end of the treatment period, whole cell extracts were separated by SDS-polyacrylamide gels, and resolved proteins were transferred to a PVDF membrane. The cleavage of endogenous substrates was assessed by immunoblotting using specific antibodies to PARP, XIAP, TcR–ζ chain, Bcl-XL, or Bcl-2.

To further determine the cleavability of caspase-8, we treated cell extracts with recombinant caspase-3 and detected similar processing of caspase-8 in extracts from sensitive and resistant Jurkat cells (Fig. 17). We also added an exogenous cytochrome c and dATP to post-mitochondria extracts of either sensitive or resistant Jurkat cells and detected processing of caspase-8 in both extracts. Such processing is presumably carried out through the activation of endogenous procaspase-3 by the apoptosome (a complex of caspase-9, APAF-1, cytochrome c and dATP) followed by subsequent activation of caspase-8 by caspase-3.

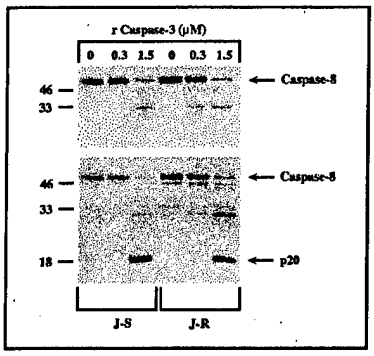


Fig. 17
Processing of endogenous caspase-8 in extracts of resistant Jurkat cells by recombinant caspase-3. Recombinant caspase-3 was added to extracts of sensitive or resistant Jurkat cells at 0.3 or 1.5 μM for 30 min at 37°C. The proteins were resolved by SDS/PAGE and probed with anti-caspase-8 mAb (5F7, UBI) (A). The membrane was stripped and reprobed with polyclonal anti-caspase-8 (Ab3, StressGen) which detects the p20 subunit (B).

These findings suggest that although caspase-8 is cleavable, this event does not occur in J-R cells. Such a lack in processing of caspase-8 could have been mediated by a caspase inhibitor, such as FLIP-L, FLIP-S (caspase-8-specific inhibitors) or a currently unknown caspase-8 inhibitor. With regard to potential function of a caspase-8 inhibitor, we did not detect any difference in either protein or gene expression of FLIP-L or FLIP-S in the two Jurkat cell lines. The possibility of inhibition of caspase-8 by as of yet an unknown inhibitor is currently being investigated.

The mitochondrial apoptotic pathway was also blocked in the J-R cells. No processing or activity of caspases was detected in these cells in response to exposure to VP-16 or staurosporin, cytotoxic drugs known to induce mitochondrial apoptotic cascade (Fig. 15). Also, no apoptosis associated alterations in the mitochondrial outer or inner membranes were detected in resistant Jurkat cells treated with VP-16. Thus, no changes in permeability transition, loss in inner membrane cardiolipin, generation of reactive oxygen species (Fig. 18), or release of cytochrome c (Fig. 19) were observed in resistant cells treated with VP-16. Furthermore, purified mitochondria from J-R, but not from J-S cells, failed to respond in cytochrome c release to treatment with truncated Bid (Fig. 20).

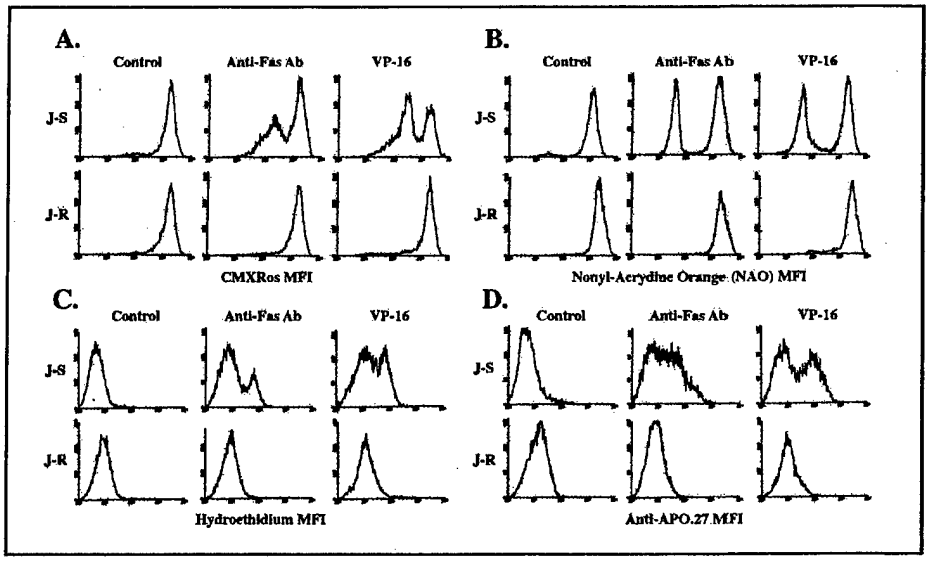


Fig. 18
Lack of apoptosis-associated alterations in mitochondria of resistant Jurkat cells treated with anti-Fas Ab or VP-16. Wild-type and resistant Jurkat cells treated with anti-Fas Ab (200 ng/ml) or VP-16 (20 μM) for 8 hr, were assessed by flow cytometry for mitochondrial changes. Staining with CMXRos (100 nM) served to assess changes in mitochondria permeability transition (A); NAO staining (100 nM) served to assess loss in mitochondria cardiolipin (B); HE staining (2 μM) served to assess the presence of ROS (C); and anti-APO.27 Ab (2.5 μg/ml) served to detect a p38 antigen specific for apoptotic mitochondria (D).

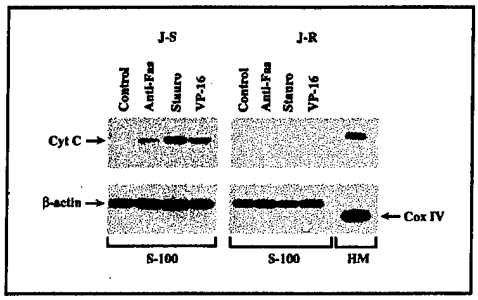


Fig. 19

Release of cytochrome c from mitochondria to the cytoplasm in response to anti-Fas Ab, staurosporin or VP-16 is blocked in resistant Jurkat cells. Wild-type and resistant Jurkat cells treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM) or VP-16 (20 μM) for 8 hr, were assessed for redistribution of cytochrome c. Following treatment, Jurkat cells were lysed and separated to a heavy membrane fraction (HM) which contains mitochondria, and a mitochondria-free S-100 fraction. The proteins were resolved by 15 % SDS/PAGE, and immunoblotted by a cytochrome c-specific Ab. Equal protein loading was ensured by protein quantification, and by reprobing the stripped membranes with anti-β-actin. Cox IV served as a marker for mitochondrial fraction. The results shown are representative of at least five independent experiments.

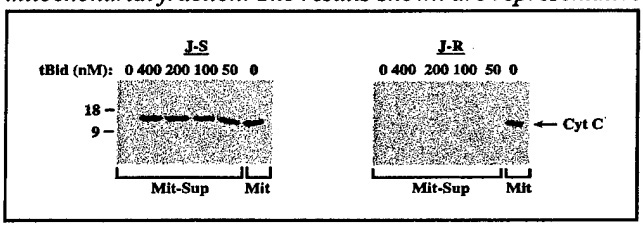


Fig. 20

Truncated Bid induces release of cytochrome c from purified mitochondria of sensitive, but not resistant Jurkat cells. Purified mitochondria from wild-type or resistant Jurkat cells were incubated with the indicated doses of recombinant tBid for 1 hr at 30°C. Mitochondria were then pelleted by centrifugation, and the resulting supernatant (Mit-Sup) was subjected to SDS/PAGE immunoblot analysis with anti-cytochrome c Ab. Input of equivalent amount of mitochondria used for each treatment was included in the analysis (Mit). The results shown are representative of at least five independent experiments.

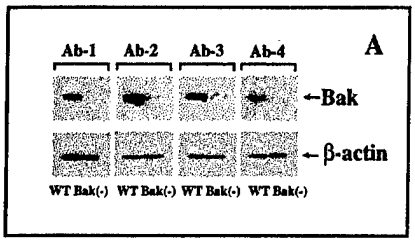
Summary and conclusions (Section III2.1)

The selection of VP-16-resistant mitochondria via elimination of Fas-susceptible cells further confirms the existence of a cross-talk between the two major apoptotic cascade initiated by death receptor signaling or mitochondrial alterations. The current knowledge does not support a possible existence of a shared regulatory component between the extrinsic and intrinsic pathways of apoptosis. It is therefore conceivable that the phenotype of the resistance to apoptosis seen in the J-R cell line is mediated by independent alterations in the two apoptotic pathways. As indicated above, despite invested effort, the mechanisms responsible for arresting the Fas cascade in J-R cells remain unknown.

III.2.2 A ROLE FOR BAK IN THE MITOCHONDRIAL APOPTOTIC CASCADE IN JURKAT T LYMPHOCYTES

Although we have not yet identified the mechanisms responsible for resistance of J-R cells to Fas-signaling, we successfully identified in these cells mechanisms involved in the resistance to mitochondrial apoptosis. As shown above, we did not detect release of cytochrome c from J-R mitochondria treated with recombinant tBid. Our results suggest that intrinsic alterations within the mitochondria are responsible for the incapability of the mitochondria to respond with release of cytochrome c. Bak and Bax are pro-apoptotic family members involved in mitochondrial response to tBid (46, 47). In order to investigate the mechanisms of resistance to mitochondrial cascade in the J-R cells, we focused our studies on expression and function Bak and Bax in J-R cells. In these studies, we utilized anti-cancer drugs as inducers of mitochondrial apoptotic events. We reasoned that such an experimental model would be the most efficient for the identification of an apoptotic block in the mitochondria of these cells. We identified Bak deficiency in J-R cells, and demonstrated that this deficiency was, in part, responsible for the observed abrogation of mitochondrial apoptotic cascade in these cells.

Deficient expression of Bak protein in J-R cells. The deficiency in Bak was determined by immunoblotting of whole cell lysates by four different anti-Bak Abs (Fig. 21A). We identified Bak-deficiency in most of the clonal cell lines obtained from the initial J-R cell line. These clonal cell lines were obtained by limiting dilutions and the results presented were obtained with clonal cell line 4 which we refer to as Bak(-) cells. Wild-type Jurkat cells obtained from ATCC served as the initial source for J-R cell line.



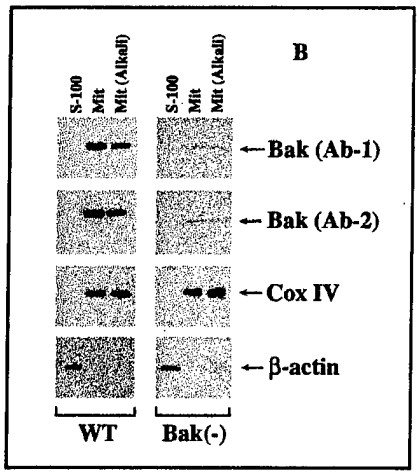


Fig. 21. Deficient expression of Bak in a clonal Jurkat cell line. (A) Wild-type (WT) or the variant Jurkat cell line, Bak(-), were incubated in 1 % NP-40 lysis buffer for 30 min at 4°C. The resultant lysates which contained both cytoplasm and mitochondria, were resolved by SDS/PAGE and assessed by immunoblotting for the presence of Bak. Four different anti-human Bak Ab were used for blotting. The membranes were stripped and reprobed for b-actin to demonstrate equal loading. (B) Expression of Bak in mitochondria of wild-type, but not in mitochondria of Bak-deficient Jurkat cells. Expression of Bak was examined in cytosol (S-100), purified mitochondria, or purified mitochondria treated with alkali to remove nonspecifically attached proteins. These cell fractions were resolved by SDS/PAGE and immunoblotted sequentially by Bak-specific Ab-1 and Ab-2. Following additional stripping, the membranes were probed with anti-Cox IV Ab, as a marker for mitochondrial fractions, and with anti-b-actin as a marker for cytosolic proteins.

To further analyze the expression of Bak, lysates of wild-type or the clonal Jurkat cells were fractionated to yield S-100 cytosol or purified mitochondria. These protein fractions were assessed for the expression of Bak by Western blot analyses. Whereas expression of Bak was detected in mitochondria of wild-type Jurkat cells, only minor expression of Bak was observed in a similar quantity of purified mitochondria from Bak-deficient cells (Fig. 21B). To assess the mitochondrial localization of Bak, purified mitochondria from either wild-type or Bak-deficient cells were treated with alkali to remove proteins nonspecifically attached to the mitochondria (48). In wild-type Jurkat cells, Bak was found to be a mitochondrial integral membrane protein, as it was detected in the pellet of alkali-treated mitochondria (Fig. 21B). Levels of protein expression of other Bcl-2 family members in Bak-deficient cells, including Bcl-2, Bcl-X_L, or Bid, were similar to those of the wild-type cell line (data not shown).

Abrogation of mitochondrial apoptotic pathways in Bak-deficient Jurkat cells. As the Bcl-2 proapoptotic family member Bak resides in the mitochondria, we examined the susceptibility of the Bak-deficient cells to apoptotic agents known to function through a variety of mechanisms (discussed below) but to commonly initiate mitochondrial pathways of apoptosis. Wild-type and Bak-deficient cells were assessed for apoptosis by various assays including the JAM (49), flow cytometry TUNEL (45), flow cytometry assessment of mitochondrial cardiolipin binding to nonyl acrydin orange (NAO) (50), and flow cytometry staining of propidium iodide and annexin V (51). Utilizing these assays we obtained similar results of resistance of the Bak-deficient cells to UV irradiation, VP-16, staurosporin, bleomycin, cisplatin, doxorubicin, 5-fluorouracil and cyclophosphamide. A part of the analyses for the resistance of the Bak-deficient cells to VP-16, bleomycin and cisplatin is shown in Figure 22A. Whereas Bak-deficient Jurkat cells remained negative to staining by FITC-annexin V or propidium iodide, high levels of apoptotic cells were detected in wild-type Jurkat cells (Fig 22A). No apoptotic cells were detected among the Bak-deficient cells at 48 or 72 hr after exposure to anticancer drugs. No processing of

caspase prodomains, as assessed by immunoblot analysis of caspase-8, -3, -2, and -7, was detected in Bak-deficient Jurkat cells treated with staurosporin or VP-16 for 14 hr (Fig. 22B). These results demonstrate that the Bak-deficient cells are absolutely resistant to apoptotic signals delivered by an array of anticancer drugs.

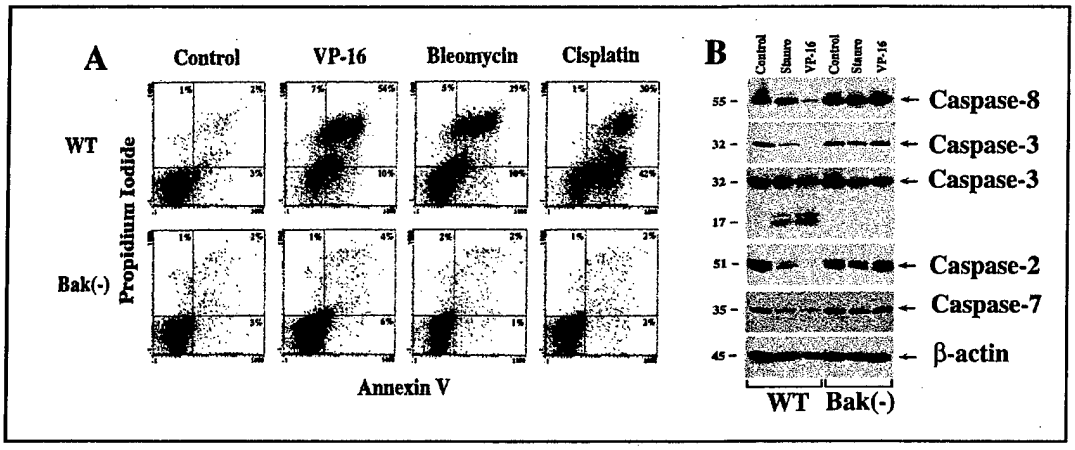


Fig. 22

Resistance of Bak-deficient Jurkat cells to anticancer drugs. (A) Flow cytometry analysis of wild-type (WT) or Bak-deficient [Bak(-)] Jurkat cells 24 hr after treatment with VP-16 (80 μM), bleomycin (300 μM) or cisplatin (100 μM). The cells were analyzed for staining by FITC-annexin V and propidium iodide. (B) Abrogation of processing of caspases in Bak-deficient Jurkat cells. Wild-type or Bak-deficient Jurkat cells were treated with staurosporin (0.5 mM) or VP-16 (20 μM) for 14 hr. Cell extracts in 0.5 % NP-40 lysis buffer were assessed by immunoblotting for the expression of the indicated prodomain caspases. Processing of caspase-3 was assessed with polyclonal Ab detecting the prodomain only (Transduction) or prodomain and subunits (PharMingen). Expression of β-actin served as an internal loading control.

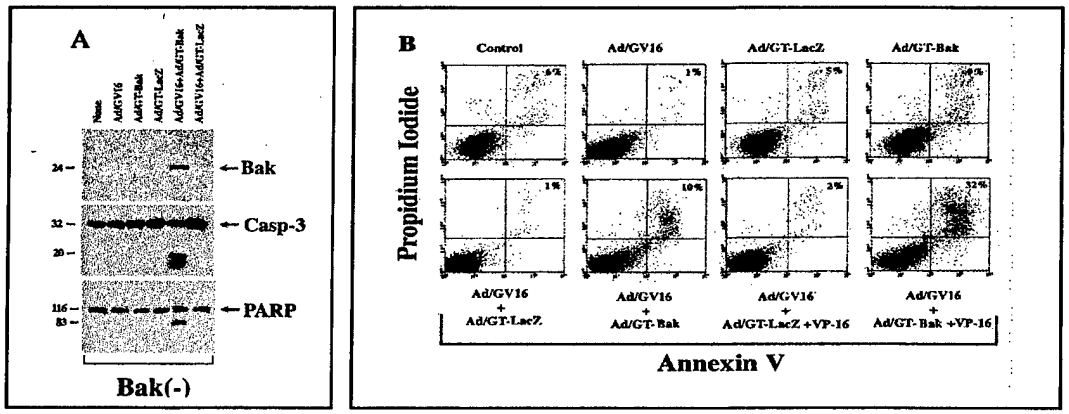


Fig. 23

Adenoviral-mediated transduction of Bak induces cell death and susceptibility to VP-16 in Bak-deficient Jurkat cells. (A) Bak expression following viral infection. Twenty four hours after infection of Bak-deficient cells with the indicated combinations of adenoviral vectors, cell extracts were assessed by immunoblotting for Bak expression. The membrane was stripped and reprobed successively for caspase-3 activation and PARP cleavage. (B) Susceptibility of Bak-deficient cells to VP-16. Bak-deficient cells that survived the first 24 hr post Bak transduction were treated with VP-16 (40 μM). Flow cytometry analysis was performed 48 hr after infection.

Transduction of Bak-deficient cells with Ad/GT-Bak and Ad/GV-16 vector. Using a binary adenoviral vector system to avoid the toxic effects of Ad/Bak on 293 packaging cells, we successfully produced large amounts of Ad/GT-Bak, whose gene product (Bak) was under the transcriptional control of the GT promoter and GAL4/GV16 fusion protein (52, 53). The binary adenoviral LacZ vector system (Ad/GT-LacZ + Ad/GV16) was used to determine transduction efficiency. Bak expression was induced when Ad/GT-Bak + Ad/GV16 were administered, but not when Ad/GT-LacZ + Ad/GV16 were used (Fig. 23A). As high levels of Bak expression induce rapid cell death (54, 55), we observed approximately 30% cell death in either wild-type or Bak-deficient cells at 24 hr after transfection (data

not shown). At 48 hr post infection, when the majority of the initial population of apoptotic cells detected at 24 hr has already been disintegrated, a population of cells susceptible to 24 hr treatment with VP-16 was detected in Bak-deficient cells administered with Ad/GT-Bak + Ad/GV16 vectors, but not with the Ad/LacZ+Ad/GV16 combination (Fig. 23B). It is plausible that a high level of Bak expression is associated with accelerated cell death, whereas cells with a lower level of Bak expression develop susceptibility to VP-16. Bak-mediated apoptosis was also confirmed by detection of caspase-3 activation and PARP cleavage in Bak-deficient cells infected with Ad/GT-Bak, but not in mock infected cells (Fig. 23A). These results confirm the role of Bak in the observed resistance to VP-16-mediated apoptosis.

Summary and conclusions (Section III.2.2)

Our findigs suggest that Bak plays a key role in the apoptotic machinery of cytochrome c release and thus in resistance of T cells to various inducers of mitochondrial apoptosis.

Lessons learned in Bax and Bak knockout murine models

Redundant roles for Bax and Bak during development. Bax and Bak represent proapoptotic Bcl-2 family members that are closely related (54-56). Bak is expressed in essentially all organs, suggesting that it may be a regulator of apoptosis in multiple cell types (57). Recently, Bak-deficient mice were generated by gene targeting (58). These mice failed to demonstrate observable developmental abnormalities. Bak-deficient mice were capable of reproduction, and cells isolated from them were susceptible to apoptosis induced by death receptor or stress. These findings suggested that either Bak is not an essential regulator of apoptosis or that the function of Bak is redundant with that of other proapoptotic Bcl-2 family members. Bax-deficient mice are also viable, demonstrating that Bax is not required for development. However, male $Bax^{-/-}$ mice are sterile and also have some neural abnormalities (59). Because of the limited phenotypic abnormalities of either $Bax^{-/-}$ or $Bak^{-/-}$ mice, Craig Thompson and his colleagues generated $Bax^{-/-}Bak^{-/-}$ mice (58). The majority of these mice died perinatally with fewer than 10% of the animals reaching adulthood. Recent characterization of cells from these mice for susceptibility to various inducers of apoptosis suggested that Bax and Bak represent redundant proteins involved in the regulation of apoptosis (60, 61). Murine cells lacking both Bax and Bak, but not cells lacking only one of these components were resistant to multiple apoptotic stimuli that act through disruption of mitochondrial function: staurosporin, etoposide and UV irradiation. These doubly deficient cells were completely resistant to tBid-induced cytochrome c release and also to the apoptotic activity of the BH3-only Bcl-2 proteins, Bim and Bad (60, 61).

We have detected similar resistance to staurosporin, VP-16, and UV irradiation in the Bak-deficient clonal Jurkat cell line (50). Bax protein has been reported to be present in the cytosol and mitochondria of Jurkat cells (62-64). It has also been reported that Bax in Jurkat cells translocates from the cytosol to the mitochondria in response to certain apoptotic stimuli (63, 64). These reports together with our findings regarding the requirement for Bak for the release of cytochrome c in response to VP-16 suggest that in Jurkat cells a deficiency in Bak alone led to apoptosis resistance, whereas in the murine system such resistance required a double deficiency in both Bax and Bak. To better understand the discrepancy between the two systems, we considered the possibility that the protein detected as Bax in Jurkat cells was not functional, and initiated an analysis of *bax* gene expression in Jurkat cells.

III.2.3 A role for Bax in the response of T Jurkat cells to anticancer drugs

Identification of Bax splice variants. To determine what are the major Bax mRNA splice variants transcribed in wild-type or Bak-deficient Jurkat cells, we carried out RT-PCR on poly-A^+ RNA from these cell lines. We utilized forward and reverse primers that correspond to the extreme 5' and 3' ends of the Baxα splice variant ORF, respectively. Electrophoresis of Bax PCR products for both Jurkat cell lines on a 3% analytical agarose gel exhibited an identical continuous array of bands ranging in size from ~400 to ~1000 bp (Fig. 24). However, a major band in both cell lines migrating between 400 and ~450 bp was detected. Based on the predicted size of known Bax splice variants this band corresponds to Baxδ. This splice variant lacks exon 3, which encodes the BH3 domain, but is otherwise identical to the Baxα variant.

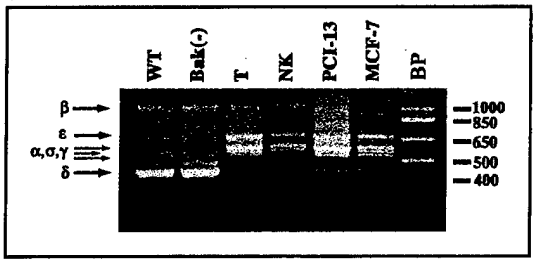


Fig. 24. Analysis of Bax mRNA splice variants. RT-PCR was carried out on RNAs isolated from 6 human cell cultures: WT and Bak-deficient Jurkat cells, peripheral blood T and NK cells, the head and neck tumor cell line PCI 13 and the breast carcinoma cell line MCF-7, using Bax-specific primers. The forward and reverse primers correspond to the extreme 5' (exon 1) and 3' (exon 6) ends, respectively, of the Baxα open reading frame. Based on the predicted size of the known Bax splice variants the splice patterns for each cell type indicate the following: Both Jurkat cell lines exhibit Baxδ (441 bp) as the major splice variant. Cultured T and NK cells present Baxγ (536 bp), Baxσ (549 bp), Baxα (588 bp) and Baxε (686 bp) as their major splice variants. Baxβ (1088 bp) appears to be present but at a lower level in these 4 cell types. The tumor cell lines PCI-13 and MCF-7 show a similar pattern to the T and NK cells but with an increased intensity in Baxδ.

The Bax mRNA pattern in either wild-type or Bak-deficient Jurkat cells was distinctly different from that of human peripheral blood T or NK cells or human tumor cell lines included in the analysis. Confirmation of the identity of the major band in Jurkat cells as Baxδ was obtained by molecular TA cloning of the gel purified band into the mammalian expression vector pCR3.1. Automated DNA sequence analysis of both strands of the cDNA insert using the primers T7 5'-TAATACGACTCACTATAGGG-3' (forward primer, corresponds to the T7 promoter region in pCR3.1) and BGHR 5'-TAGAAGGCACAGTCGAGG-3' (reverse primer, corresponds to BGHR sequence in pCR3.1) allowed for sequence confirmation. These results suggest that both wild-type and Bak-deficient Jurkat cells used in our studies may have a relative deficiency in the expression of Baxα with a major shift toward expression of Baxδ, a BH3-deleted isoform, whose function in apoptosis has not yet been investigated.

Determination of *bax* gene sequence. It has been reported that colon carcinomas (44) as well as human hematopoietic tumors (45) can contain mismatch repair (MMR) deficient cells which results in the mutation of mononucleotide tracts in their DNA. Thus, the Bax gene which contains a G_8 tract in exon 3 (nucleotides 114 to 121) is often mutated in one of its alleles by the addition or deletion of a G nucleotide. This mutation results in a translational frame shift of any Bax splice variant that contains exon 3, and therefore, prevents expression of functional Bax from that allele. To determine if wild-type or Bak-deficient Jurkat cells possessed the MMR phenotype we carried out RT-PCR on Jurkat RNAs using the forward primer Bax-6 5'-TTTCATCCAGGATCGAGCAG-3' (corresponds to extreme 5' end of exon 3) and the reverse primer Bax-5 5'-GCCTCAGCCCATCTTCTT-3' that corresponds to the extreme 3' (COOH-terminal) end of the Baxα splice variant ORF. A major band of ~500 bp was extracted from an agarose gel for both the wild-type and Bak-deficient RNAs and subcloned into the vector pCR3.1. DNA sequence analysis carried out on a total of twenty randomly picked clones from

both cell line libraries failed to detect the wild-type allele. The sequence of these clones was otherwise identical to Baxα and -γ variants. Our analysis of the *bax* gene sequence was performed with Jurkat cells obtained from ATCC just prior to the study. Thus, the lack of the wild-type *bax* gene characterizes the source cells and does not represent an alteration acquired during recent cell passages. These results suggest that the Jurkat cells obtained from ATCC as well as our Bak-deficient cells are deficient in the wild-type Baxα protein. Furthermore, any splice variant of the *bax* gene in Jurkat cells that contains exon 3, which encodes the BH3 domain, will be mutated.

We are aware that multiple studies have reported the expression of Bax in Jurkat cells (62-65). The N-20 anti-Bax Ab (Santa Cruz Inc.) is capable of detecting Baxδ as an *in vitro* translated product or as a band of ~16 kDa in Jurkat cell extract (our preliminary data, not shown). Thus, the detected protein ban may either be a non-specific protein or the Baxδ isoform. However, as described above, this BH3-deleted Bax isoform is not expected to have a similar pro-apoptotic function as that of wild-type Baxα.

Summary and conclusions (Section III.2.3)
The results described above suggest that the ATCC wild-type Jurkat cells are Baxα-deficient, thus the Bak-deficient clonal cell line we selected from the wild-type Jurkat cells carries a double deficiency in both Bax and Bakα.

Do Bax and Bak serve redundant roles in the execution of mitochondrial apoptosis? As suggested by the murine $Bax^{-/-}Bak^{-/-}$ model, Bax and Bak serve a redundant role in the response to certain anticancer drugs (58, 60). However several studies have indicated that the function of these two pro-apoptotic proteins is not completely redundant. A recent study by Vogelstein's group (66) suggested that the redundancy between Bax and Bak did not apply in human colorectal carcinoma cells, as tumor cells targeted for deficiency in Bax, but not for Bak, were resistant to nonsteroidal anti-inflammatory drugs (NSAID). Thus, in these cells, the presence of Bak (confirmed in our analysis of these $Bax^{-/-}$ HCT116 cells) could not compensate for the absence of Bax, indicating a unique role for Bax in the apoptotic response to NSAID. Also, an apoptotic response to TRAIL of these colon carcinoma cells has been demonstrated to be Bax-dependent, and was not compensated for by the endogenous levels of Bak (67, 68). These reports suggest that although the mitochondrial roles of Bax and Bak may be redundant in certain types of cells and in response to certain apoptotic stimuli, they may manifest differential functions in other systems.

Differentially acquired susceptibility to VP-16 in Bax/Bak-deficient Jurkat cells upon gene transduction of *bax* or *bak*. To determine whether either Bax or Bak is sufficient for signaling through the mitochondria in response to cytotoxic drugs, we initiated studies to induce the expression of Bax in Bax/Bak-deficient cells utilizing a similar approach to the one described above for Bak. Utilizing a binary adenoviral system for Bax, we successfully transduced the *bax* gene into Bax/Bak-deficient cells (Fig. 25). Similar to Bak, transduction of Bax induced an apoptotic response, as demonstrated by partial processing of procaspase-3 and PARP (Fig. 25A). However, in contrast to transduction of Bak, transduced expression of Bax did not result in an acquired susceptibility of these cells to VP-16-mediated apoptosis (Fig. 25B). These findings are different from those obtained in Bax/Bak knockout murine embryonic fibroblasts (MEF), and may relate to the presence of p53 in the murine model and to its absence in the Jurkat T leukemic cells.

Nevertheless, these preliminary results suggest that Bax/Bak-deficient Jurkat cells acquire differential susceptibility to VP-16 upon transduction of the *bax* or the *bak* genes.

It appears that in Jurkat cells (which are p53-null) Bax and Bak serve differential roles in the cell response to the DNA damage sustained in the presence of VP-16.

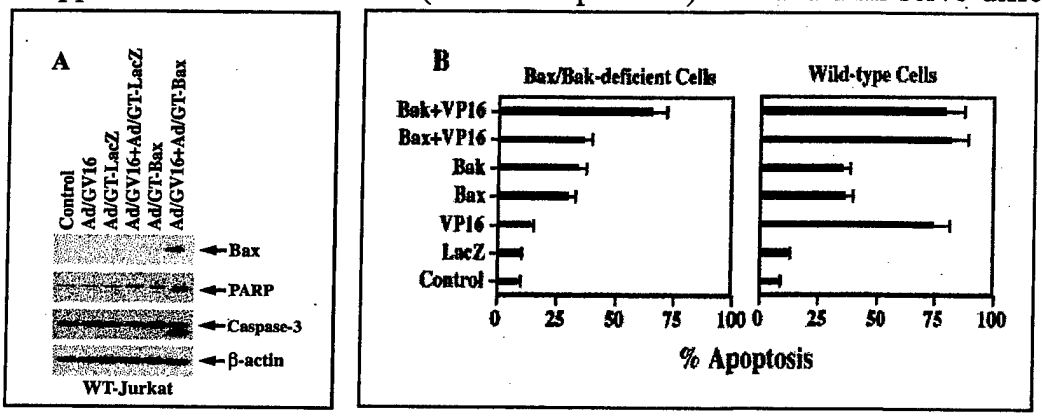


Fig. 25. *(A) Restored expression of Baxa in wild-type Jurkat cells following adenoviral transduction of this gene. Expression was assessed by immunoblotting 24 hr after exposure of the cells to the adenoviral vectors. The membrane was stripped and reprobed with anti-PARP and anti-caspase-3 to demonstrate that the transfected gene (Bax) was functional. The membrane was stripped again and reprobed with anti-β-actin Ab to demonstrate equal loading. (B) Differential susceptibility to VP-16 of Bax/Bak-deficient cells transduced with the Bax or Bak genes. The cells were exposed to the adenoviral vectors and to VP-16 at the same time. The level of apoptosis was assessed by counting live cells 16 hr post transduction. Results of one representative experiment of three performed are presented. The error bars represent the standard deviations of three independent counts of each sample.*

Summary and conclusions (Section III.2.3)

Our findings suggest that the multidomain proapoptotic Bcl-2 family members, Bax and Bak, carry distinct roles in certain cell types and in response to specific apoptotic stimuli. The resistance of the clonal Jurkat cells to BrCA-mediated apoptosis may relate to the deficiency in Bak, Bax or both. We have performed experiments to determine whether J-R cells transduced with either Bak or Bax acquire susceptibility to apoptosis mediated by interaction with BrCA cells. However, results of these studies were inconclusive because of the background apoptosis mediated by transfection of a 'killer' gene, such as Bax or Bak. We plan to develop inducible transfection systems for Bax and Bak that are expected to overcome the background apoptosis observed (69). In addition, we are developing RNAi approach to downregulate the expression of Bax and Bak (70-72). Such approach is expected to enable to further target the molecular mechanisms underlying resistance of T lymphocytes to BrCA-mediated apoptosis.

III.3.3 CYTOKINE-MEDIATED PROTECTION OF T LYMPHOCYTES FROM BRCA-MEDIATED APOPTOSIS

As demonstrated in Sections II.3 and III.1, endogenous inhibitors of apoptosis such as FLIP, anti-apoptotic Bcl-2 family members and IAPs interfere with the level of apoptosis induced in T lymphocytes through interaction with BrCA. The levels of expression of certain endogenous inhibitors of apoptosis have been reported to increase in the presence of cytokines. We, therefore, investigated whether cytokines known to modulate function of T cells will have protective effects against apoptosis induced by BrCA. ^{3}H-labeled Jurkat cells were incubated with BrCA cells in the presence of various doses of IL-2, IL-7 or IL-12. We detected significant reduction in the loss of DNA in Jurkat cells in the presence of each of these cytokines (Fig. 26A). Similar results were also obtained in activated PBL co-incubated with BrCA cells in the presence of these cytokines (Fig. 2B). We currently investigate the

endogenous mechanisms involved in the observed protection. In preliminary results we detected an increased expression of Bcl-XL in Jurkat cells treated with IL-12 (data not shown).

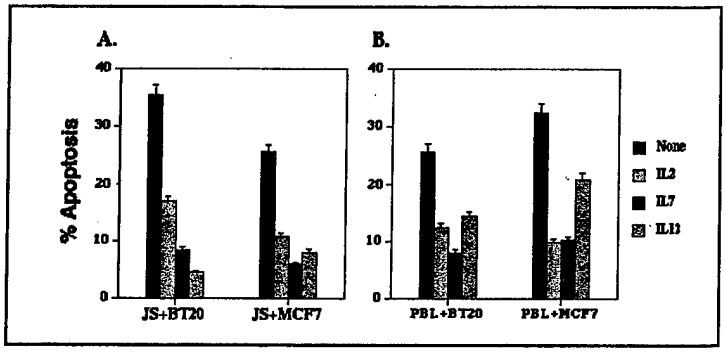


Fig. 26

Cytokine protection of T lymphocytes from BrCA-induced apoptosis. BrCA cell lines were co-incubated with [^{3}H]TdR-labeled Jurkat cells (A) or with ^{3}H]TdR-labeled ConA-activated PBL (48h) in the presence or absence of IL-2, IL-7 or IL-12 (100 U/ml each) for 16h. Loss in labeled DNA was used as a measure of apoptosis. The error bars represent the SEM of 8 replicates.

Summary and conclusions (Section III.3)

Our results suggest that exposure of T lymphocytes to the cytokines IL-2, IL-7 or IL-12 partially protects these cells from BrCA-induced apoptosis. Additional studies are required to determine the molecular mechanisms responsible for the observed protection.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstration of the involvement of the death receptors, Fas and TRAIL-Rs, in BrCA-induced apoptosis of T lymphocytes
- Demonstration of the involvement of the mitochondrial apoptotic cascade in BrCA-induced apoptosis of lymphocytes
- Partial blocking of BrCA-induced apoptosis of T cells by endogenous inhibitors of the Fas death receptor pathway, including CrmA, FLIP-L or FLIP-S
- Partial blocking of BrCA-induced apoptosis of T cells by BCL-XL, an endogenous inhibitor of the mitochondrial apoptotic cascade
- A complete blockade of BrCA-induced apoptosis of T cells requires the inhibition of the two major apoptotic pathways
- Demonstration of protection of T lymphocytes from BrCA-induced apoptosis by the cytokines, IL-2, IL-7, and IL-12
- Identification of hILP/XIAP as a substrate of caspases in apoptotic T lymphocytes
- Identification of caspase-3 and –7 as mediators of this cleavage
- Mapping of the hILP cleavage site to aspartic acid-242
- Detection of the hILP cleavage product in T cells undergoing AICD or co-incubated with BrCA cells
- Detection of the hILP cleavage product *in vivo* in TAL (OvCA)
- Identification of an apoptosis resistant clonal Jurkat cell line in which both the Fas death receptor pathway and the mitochondrial cascade are blocked
- Demonstration that deficiencies in Bak and/or Bax efficiently block the mitochondrial apoptotic cascade induced by various apoptotic agents

REPORTABLE OUTCOMES

1. Wang, G. Q., Gastman, B. R., Wieckowski, E., Goldstein, L. A., Gambotto, A., Kim, T. H., Fang, B., Rabinovitz, A., Yin, X. M., and Rabinowich, H. A role for mitochondrial Bak in apoptotic response to anticancer drugs. J Biol Chem. *276:* 34307-34317, 2001.

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These manuscripts acknowledge the DOD support. Results obtained in the tumor models used in manuscepts 2 and 4 (head and neck cancer and OvCA) had many similarities to results obtained later with BrCA.

CONCLUSIONS

Our studies demonstrated that the two known apoptotic pathways of apoptosis, the death receptor cascade and the mitochondrial cascade are involved in BrCA-induced T cell death. Overexpression of endogenous inhibitors specific to each of the apoptotic pathways had significant, but partial, reducing effect on the level of the apoptosis observed. Therefore, efficient inhibition of T cell death in the tumor microenvironment would require the blocking of the two major apoptotic pathways. Indeed, the clonal Jurkat T cell line, in which both apoptotic pathways were blocked, demonstrated a complete resistance to tumor-induced apoptosis. Elucidation of the molecular mechanisms responsible for the block(s) in the apoptotic machinery of this T cell line may help identify an efficient target for therapeutic intervention aiming at protecting effector T cells from premature apoptotic death at the tumor site.

The anti-apoptotic protein XIAP/hILP undergoes caspase-mediated cleavage in T lymphocytes interacting with BrCA. These observations not only confirm our hypothesis that immune effector cells are induced to apoptose in the tumor microenvironment, but also suggest an intracellular mechanism which renders the cell susceptible to apoptosis. Degradation of XIAP/hILP is still a very hot and important issue in apoptosis research. In addition to being cleaved by caspases, new evidence suggests that it may be also subjected to degradation by other proteolytic mechanisms. We and other leading groups in the apoptosis research are pursuing this issue.

The block in the apoptotic mitochondrial cascade in J-R clonal cell line was found to be double-deficiency in Bax and Bak. We identified the molecular mechanism responsible for the deficiency in wild-type Bax (Baxα). The mechanism responsible for the down-regulated expression of Bak remains unknown. In development, Bax and Bak appears to have redundant roles (58, 60, 61). However, we obtained evidence that they may have differential roles in response to specific inducers of apoptosis (manuscript submitted). We currently do not know whether deficiency in Bax or Bak is sufficient to block in the mitochondrial cascade induced by BrCA. In the future, we plan to apply RNAi approach to specifically down-regulate Bax or Bak in T lymphocytes, and determine the subsequent level of susceptibility of the treated cells to BrCA-induced apoptosis. Such an RNAi approach may be useful in reducing tumor-mediated apoptosis of T cells used for adoptive immunotherapy (73).

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APPENDIX

Hannah Rabinowich, Ph.D.

Four papers acknowledging the DOD support

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Advances in Brief

Tumor-induced Apoptosis of T Cells: Amplification by a Mitochondrial Cascade[1]

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Abstract

We have recently reported that apoptosis of T cells induced by squamous cell carcinoma of the head and neck (SCCHN) is partly Fas dependent. This tumor-induced T-cell death is mediated by the activities of caspase-8 and caspase-3 and is partially inhibited by antibodies to either Fas or Fas ligand. We report here that in contrast to apoptosis induced by agonistic anti-Fas antibody (Ab), the tumor-induced apoptotic cascade in Jurkat cells is significantly amplified by a mitochondrial loop. The involvement of mitochondria in tumor-induced apoptosis of T cells was demonstrated by changes in mitochondrial permeability transition as assessed by 3,3′-dihexiloxadicarbocyanine staining, by cleavage of cytosolic BID and its translocation to the mitochondria, by release of cytochrome *c* to the cytosol, and by the presence of active subunits of caspase-9 in Jurkat T cells cocultured with tumor cells. To further elucidate the significance of mitochondria in tumor-induced T-cell death, we investigated the effects of various inhibitors of the mitochondrial pathway. Specific antioxidants, as well as two inhibitors of mitochondria permeability transition, bongkrekic acid and cyclosporin A, significantly blocked the DNA degradation induced in Jurkat T cells by SCCHN cells. However, these inhibitors had no effect on cells triggered by anti-Fas Ab. Furthermore, a cell-permeable inhibitor of caspase-9, Ac-LEHD.CHO, which did not inhibit T-cell apoptosis induced by anti-Fas Ab, markedly inhibited apoptosis induced by etoposide or by coculture of Jurkat with SCCHN cells. These findings demonstrate that apoptotic cascades induced in Jurkat T lymphocytes by anti-Fas Ab or tumor cells are differentially susceptible to a panel of inhibitors of mitochondrial apoptotic events. It appears that besides the Fas-mediated pathway, additional mitochondria-dependent cascades are involved in apoptosis of tumor-associated lymphocytes. Inhibition of mitochondria-dependent cascades of caspase activation should be considered to enhance the success of immunotherapy or vaccination protocols in cancer.

Introduction

Recent studies suggest that human carcinoma cells of various origins can activate intrinsic programmed cell death in lymphocytes interacting with the tumor *in situ* and *in vitro* (1–3). This tumor-induced apoptosis of lymphocytes may have important implications for the success of therapeutic regimens, including vaccination strategies (4). Because tumor-induced apoptosis of lymphocytes may be mediated by an array of death receptors coexpressed on T cells or by tumor-derived soluble factors, it is important to characterize those intracellular events that may be potential targets for therapeutic intervention to minimize T-cell apoptosis. The caspases, a family of cysteine proteases, play critical roles in the execution phase of apoptosis and are responsible for many of the biochemical and morphological changes associated with apoptosis (5–7). Caspase-8 has been identified as the most apical caspase in apoptosis induced by several death receptors, including Fas and TNFR1[3] (8). Fas-associated death domain is recruited directly to ligated Fas or indirectly to ligated TNFR1, resulting in recruitment and autoactivation of caspase-8. Active caspase-8 cleaves and activates downstream caspases, initiating the caspase cascade. Caspase-9 has been proposed as the initiating caspase in a pathway of apoptosis that is death receptor independent (9, 10). In the presence of dATP and cytochrome *c*, the long NH_2-terminal domain of caspase-9 interacts with APAF-1, resulting in activation of caspase-9. Active caspase-9 can then activate the effector caspase-3, -6, and -7 (10, 11). Thus, there are at least two major mechanisms by which a caspase cascade may be initiated: (*a*) one involving capase-8; and (*b*) the other involving caspase-9 as the most apical caspase.

These two basic pathways of caspase activation allow predictions as to how the apoptotic cascade is regulated under different circumstances. It is expected that various inhibitors of apoptosis, including Bcl-2 family members, CrmA, FLICE-inhibitory protein, or inhibitors of apoptosis, which target different caspases or intracellular apoptotic events, will differentially regulate the two caspase activation cascades. For example, antiapoptotic Bcl-2 family members bind to mitochondria and inhibit release of cytochrome *c* (12, 13). Therefore, apoptotic signaling via death receptors should be resistant to Bcl-2 (14). However, it seems that Bcl-2 and Bcl-x_L can also interfere with Fas-mediated apoptosis in cells in which the Fas/Fas-associated death domain/procaspase-8 recruitment is not efficient (15). Because Fas ligation is associated with release of cytochrome *c*, it raises the possibility of cross-talk between the two basic pathways. Recently a mechanism of cross-talk between caspase-8 and caspase-9 via mitochondria was identified (9, 16, 17). BID, a proapoptotic member of the Bcl-2 family, is cleaved by caspase-8, and its COOH-terminal fragment translocates to the mitochondria and triggers release of cytochrome *c* (9, 16, 17). Depletion of BID from cytosolic extracts disrupts the ability of caspase-8 to trigger cytochrome *c* release *in vitro* (17).

The current study investigated intracellular apoptotic events in Jurkat T cells interacting with SCCHN. The intracellular effector molecules involved in execution of tumor-induced death of lymphocytes, which might serve as potential targets for inhibition of apoptosis, have not yet been elucidated. Our recent studies (18, 19) demonstrated that apoptosis induced in T lymphocytes by tumor cells was, in part, Fas mediated and involved activation of caspase-8 and -3.

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[3] The abbreviations used are: TNFR, tumor necrosis factor receptor; BA, bongkrekic acid; CsA, cyclosporin A; DiOC6(3), 3,3′-dihexiloxadicarbocyanine; ROS, reactive oxygen species; SCCHN, squamous cell carcinoma of the head and neck; Ab, antibody; CMXRos, chloromethyl-X-rosamine; GAMIg, goat antimouse immunoglobulin; DPI, diphenyleneiodonium chloride; NAC, *N*-acetyl-L-cysteine; mAb, monoclonal Ab; dThd, thymidine; HM, heavy membrane; PT, permeability transition; ANT, adenine nucleotide translocator.

In the present study, we investigated the role of a mitochondrial cascade and its significance in SCCHN-induced apoptosis of Jurkat T lymphocytes. Our findings suggest that in contrast to Fas-mediated apoptosis of Jurkat cells, which is mitochondria independent, mitochondria have a significant effector role in tumor-induced cell death of interacting T cells.

Materials and Methods

Reagents. Agonistic anti-Fas Ab (CH-11; IgM) was purchased from Upstate Biotechnology (Lake Placid, NY), rabbit anti-caspase-9 Ab (clone H-83) was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-cytochrome *c* was from PharMingen (San Diego, CA), and anti-cytochrome *c* oxidase was from Molecular Probes (Eugene, OR). Rabbit anti-BID polyclonal Ab used has been described previously (20). The caspase-9 inhibitor, Ac-LEHD.CHO, was purchased from NovaBiochem (San Diego, CA), DiOC6(3) and CMXRos were from Molecular Probes, and GAMIg-conjugated magnetic beads were from PerSeptive Diagnostics (Cambridge, MA). Staurosporin, etoposide (VP-16), CsA, diamide, and the antioxidants DPI and NAC were purchased from Sigma (St. Louis, MO). BA was a generous gift from Dr. J. A. Duine (University of Delft, Delft, the Netherlands). Anti-$\alpha_6\beta_4$ mAb (A9) was a generous gift from Dr. T. E. Carey (University of Michigan Cancer Center, Ann Arbor, MI).

Cell Lines. The human Jurkat T-cell leukemia cell line was grown in RPMI 1640 supplemented with 10% FCS, 50 mM HEPES buffer, and 2 mM L-glutamine (Life Technologies, Inc.). This Jurkat cell line (Ju-S) is sensitive to a variety of apoptosis-inducing agents, including anti-Fas Ab, VP-16, and staurosporin. A Jurkat cell line (Ju-R) resistant to apoptosis induced by either anti-Fas Ab or VP-16 was used as a negative control (3). The previously described SCCHN cell lines PCI-13, PCI-52, OSC-19, SCC-68, and SCC-74 were grown in DMEM supplemented with 10% FCS, 50 mM HEPES buffer, and 2 mM L-glutamine (4, 18, 19).

Lymphocytes and Tumor Cells Coculture. To induce apoptosis or apoptosis-related changes in lymphocytes, SCCHN cell lines were coincubated with Jurkat cells for 16–24 h at a tumor:lymphocyte cell ratio ranging from 20:1 to 80:1. To assess processing of intracellular proteins, Jurkat cells were negatively selected by removal of SCCHN cells using epithelial-specific $\alpha_6\beta_4$ mAb (A9) and GAMIg-conjugated magnetic beads. To this end, cocultures of SCCHN and Jurkat cells were incubated with A9 mAb at 50 μg/10^7 cells/ml on ice for 1 h. The cells were washed three times in cold medium and subjected to two cycles of incubation with GAMIg-coated magnetic beads (30:1, beads: cell ratio). As assessed by flow cytometry, tumor cells were efficiently removed because 99% of the negatively selected cells did not bind anti-$\alpha_6\beta_4$ integrin mAb. Jurkat cells cocultured with normal skin fibroblasts or triggered by agonistic anti-Fas Ab (CH-11; 200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) served as controls. Inhibitors of apoptosis, including BA, CsA, caspase-9 inhibitors, or antioxidants, were added to Jurkat cells 2 h before the induction of apoptosis. Stock solutions of drugs in DMSO were stored at −20°C. Control cells received solvent alone. The final concentration of DMSO solvent in the culture medium never exceeded 1% (v/v), which was nontoxic to the cells.

Analysis of Apoptosis. DNA fragmentation was assessed by the JAM assay, in which loss of [^{3}H]dThd-labeled DNA was measured (3). DNA labeling of Jurkat target cells was performed by incubation of the cells in the presence of 5 μCi/ml [^{3}H]dThd for 18–24 h at 37°C. Tumor cells were cocultured with [^{3}H]dThd-labeled target cells for 16 h at 37°C at tumor: lymphocyte or normal fibroblast:lymphocyte cell ratios ranging from 10:1 to 80:1. At the end of the coincubation period, the cells were harvested (Mach IIM; TOMTEC) onto glass fiber filters. The radioactivity of unfragmented DNA retained on the glass fiber filters was measured by liquid scintillation counting. Specific DNA fragmentation was calculated according to the following formula: percentage of specific DNA fragmentation = $100 \times (S - E)/S$, where S represents retained DNA in the absence of effector cells (spontaneous), and E represents experimentally retained DNA in the presence of tumor (effector) cells.

Apoptosis-associated alterations in Jurkat cells were also evaluated by flow cytometry analysis of permeabilized cells stained with the potential-sensitive dye DiOC6(3), which is accumulated in mitochondria (21). Loss in DiOC6(3) staining indicates disruption of the mitochondrial inner transmembrane potential ($\Delta\psi$m) associated with apoptosis (22). Cells were first stained for DiOC6(3) (40 nM, 15 min at 37°C) and then stained without fixation by phycoerythrin-conjugated anti-CD3 Ab. CD3$^+$ cells were gated to assess mitochondrial staining by DiOC6(3).

Subcellular Fractionation. After induction of apoptosis, Jurkat cells were harvested in isotonic mitochondrial buffer (20 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin) and Dounce homogenized by 15–20 strokes. Samples were transferred to Eppendorf centrifuge tubes and centrifuged at 500 × *g* for 5 min at 4°C to eliminate nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 × *g* for 30 min at 4°C to obtain the HM pellet. The supernatant was further centrifuged at 100,000 × *g* for 1 h at 4°C to yield the final soluble cytosolic fraction, S100. HM and S100 subcellular fractions were assessed for the presence of cytochrome *c* or cytochrome *c* oxidase by Western blot analyses.

Analysis of Protein Expression by Western Blotting. After apoptosis-inducing treatment, cells were washed and lysed in lysis buffer [1% NP40, 20 mM Tris (base pH 7.4), 137 mM NaCl, 10% glycerol, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml aprotinin]. Cell lysates cleared of debris and nuclei were resolved on 15% SDS gels and transferred to a polyvinylidene difluoride membrane (Immobillon P; Milipore, Bredford, MA) as described previously (18). After probing with specific primary antibodies, the immunoreactive proteins were visualized using horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence (Pierce, Rockford, IL). Equal protein loading was routinely confirmed by stripping the Ab off the membrane and probing with anti-β-actin (Sigma). At times, the presence of an unspecific band equivalently expressed in all specimens served as a loading control. All immunoblots presented were confirmed to have equal protein loading.

Subcellular Localization of BID and Cellular Organelles. Jurkat cells, either controls or those treated with staurosporin or anti-Fas Ab or coincubated with tumor cells, were stained with CMXRos (200 nM) for 30 min at 37°C. After washing the cells with PBS, the cytospins of the cells were prepared and fixed in 2% paraformaldehyde for 10 min at room temperature. The cells were then washed five times in PBS. After permeabilization by 0.1% Triton X-100 in PBS at 4°C for 15 min, the cells were washed three times with PBS and three times with 0.5% BSA and 0.15% glycine (buffer A). The cytospins were then treated with a 4% dilution of goat serum in buffer A for 1 h at room temperature. After five washes in buffer A, the cells were treated with rabbit anti-BID and mouse anti-CD3 at 4°C overnight. After five additional washes in PBS/BSA, FITC-conjugated goat antirabbit Ab and biotin-conjugated goat antimouse Ab were added for 1 h at room temperature. After five washes, cytospins were treated with Cy5-strepavidin for 1 h, followed by nuclei staining with Hoechst (1 μg/ml), and mounted using Gelvatol (Monsanto, St. Louis, MO). The cytospins were then observed by fluorescence microscopy using a Zeiss Axiovert 13S microscope equipped with a Hammamatsu Orca camera and filter sets for Hoescht, FITC, rhodamine (to detect CMXRos), and Cy5. Images were collected using MetaMorph (Universal Imaging Corp.).[4]

Results

Mitochondria Involvement in Tumor-induced Apoptosis of T Cells. To assess the role of mitochondria in tumor-induced apoptosis of T cells, we first analyzed changes in mitochondria pore PT using the mitochondria dye DiOC6(3). After coincubation with tumor cells, CD3$^+$ Jurkat cells were assessed for loss in DiOC6(3) uptake, which is indicative of altered pore PT. Changes in the PT were induced in Jurkat cells during a 16-h coculture with SCCHN cells, including PCI-13 (Fig. 1), PCI-52, OSC-19, SCC-68, or SCC-74 (data not shown). No changes were detected in DiOC6(3) staining in Jurkat cells cocultured with control fibroblasts (data not shown).

Cleavage of BID and Translocation to the Mitochondria in T Cells Interacting with Tumor Cells. BID, a BH3 domain-containing Bcl-2 family member, has recently been identified as a factor that relays signals from cell surface death receptors to the mitochondria (16, 17, 23). A COOH-terminal fragment of BID cleaved by caspase

[4] www.image1.com.

8 translocates to the mitochondria and triggers cytochrome *c* release (17). To investigate the involvement of BID in tumor-induced apoptosis of lymphocytes, the presence of the BID proform or its cleaved fragments was examined by Western blotting. As shown in Fig. 2, BID was cleaved in apoptosis-sensitive (Ju-S) but not in apoptosis-resistant (Ju-R) Jurkat cells cocultured with tumor cells. In Jurkat cells treated with agonistic anti-Fas Ab, most of the BID proform (M_r 25,000) was processed, and two cleaved fragments of M_r 13,000 and M_r 15,000 were detected. In Jurkat cells coincubated with tumor cells, the BID proform was partly processed, and only one cleavage fragment was detected. The difference in BID processing may relate to the differential apoptotic signal delivered by agonistic Fas Ab *versus* tumor cells.

To determine whether BID translocates to the mitochondria in Jurkat cells coincubated with tumor cells, the cocultured cells were stained (Fig. 3) with anti-CD3-conjugated to Cy5 (*magenta*) to detect T cells, with Hoechst (*blue*) to assess nuclear morphology, with BID-specific Ab visualized by FITC-tagged secondary Ab, and with the MitoTracker dye CMXRos (*red/orange*). Using this image analysis procedure, multiple layers are viewed simultaneously, and only when three-dimensional colocalization occurs is a color shift detected. In control nonapoptotic Jurkat cells, BID was detected in the cytoplasm and in some nucleoli (Fig. 3*A*, *middle panel*). CMXRos-stained mitochondria in control cells were also detected in the cytoplasm, but with no colocalization with BID (Fig. 3*A*, *right panel, red/orange*). In Jurkat cells coincubated with tumor cells, BID was detected in the cytoplasm, in colocalization with the mitochondria, as assessed by the color shift to *yellow* (Fig. 3*B*). CD3 staining (*magenta*) served to distinguish lymphocytes from surrounding tumor cells. Similar translocation was observed in Jurkat cells treated with staurosporin (Fig. 3*C*) or agonistic anti-Fas Ab (Fig. 3*D*), which served as positive controls. Thus, control mitochondria stained with CMXRos (*red/orange*; Fig. 3*A*, *right panel*) shifted to a *bright yellow* color after translocation of and colocalization with BID-FITC (Fig. 3, *B–D, right panels*). These results demonstrate that BID translocation to the mitochondria occurs under a variety of apoptotic stimuli, including that initiated by tumor cells.

A Role for Cytochrome *c* in Tumor-induced T-cell Apoptosis. To directly investigate the role of mitochondria in tumor-induced apoptosis of lymphocytes, we examined release of cytochrome *c* to the cytosol in Jurkat cells after coculture with tumor cells for 4 h. As shown in Fig. 4, release of cytochrome *c* to the cytosol (S100 fraction) was detected in Jurkat cells stimulated with anti-Fas Ab (Fig. 4*A*) or coincubated with tumor cells (Fig. 4*B*). In control untreated cells, cytochrome *c* was detected exclusively in the HM fraction containing the mitochondria. The observed release of cytochrome *c* was apoptosis mediated because there was no release of the inner mitochondrial membrane enzyme, cytochrome *c* oxidase (Fig. 4, *A* and *B*). No release of cytochrome *c* was observed in the apoptosis-resistant Jurkat cell line coincubated with tumor cells, consistent with these cells being resistant to both the Fas death receptor pathway and a mitochondrial pathway (VP-16).[5] Taken together, these results suggest that the mitochondria are involved in tumor-induced apoptosis of T cells, and the release of cytochrome *c* to the cytosol may initiate a mitochondrial pathway of apoptosis.

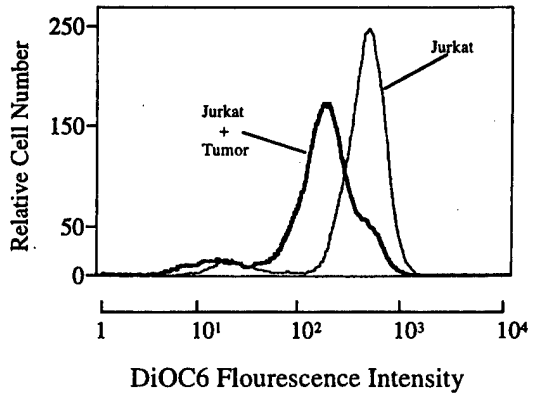

Fig. 1. Tumor-induced apoptosis of Jurkat cells as assessed by loss of DiOC6(3) staining of the mitochondria. Jurkat cells incubated in medium alone or with tumor cells (tumor:lymphocyte cell ratio of 40:1) for 16 h were stained with DiOC6(3) (40 nM, 15 min, 37°C) and then, without fixation, stained by phycoerythrin-conjugated anti-CD3 on ice. DiOC6(3) staining was assessed in $CD3^+$ cells. Jurkat cells coincubated with normal fibroblasts were similar in DiOC6(3) staining to Jurkat control cells. Results of one representative experiment of at least five performed with PCI-13 and other SCCHN cell lines are shown.

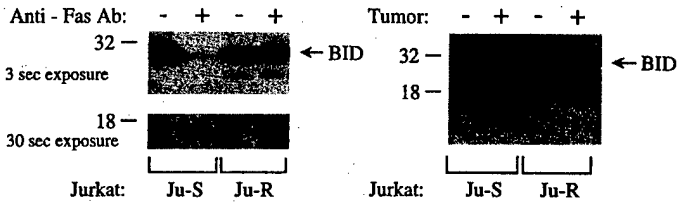

Fig. 2. Cleavage of BID in Jurkat cells coincubated with tumor cells or treated with agonistic anti-Fas Ab. Jurkat cells were treated with anti-Fas Ab (CH-11, 200 ng/ml) or cocultured with tumor cells (tumor:lymphocyte cell ratio of 20:1) for 16 h. Jurkat cells were negatively selected by removal of tumor cells as described in "Materials and Methods." Whole cell lysates of Jurkat cells were resolved on 15% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Rabbit anti-BID polyclonal antiserum was used for probing.

Activation of Caspase-9 in Tumor-induced Apoptosis of T Cells. In cells treated with chemotherapeutic agents, release of cytochrome *c* from the mitochondria has been shown to cause activation of caspase-9. Because coincubation of Jurkat cells with SCCHN cells induced release of cytochrome *c* to T-cell cytosol, we examined whether the released cytochrome *c* was sufficient to activate caspase-9. Activation of caspase-9 in Jurkat cells coincubated with tumor cells was demonstrated by detection of its active subunits by immunoblotting (Fig. 5). Cleaved products of caspase-9 were also detected in Jurkat cells triggered by either anti-Fas Ab, staurosporin, or VP-16.

Effects of Inhibitors of the Apoptotic Mitochondrial Pathway on Tumor-induced T-cell Death. To elucidate the significance of the mitochondria in tumor-induced T-cell death, we investigated the effects of various inhibitors of mitochondrial pathways. ROS or oxidants are formed in the mitochondria but become toxic when present in excessive amounts, causing oxidative damage (24). To assess the significance of a redox imbalance, we used the antioxidants DPI (25 μM), a specific inhibitor of flavin-dependent oxidoreductase (25), and NAC (10 mM), a thiol antioxidant (26). Before incubation with tumor cells or agonistic anti-Fas Ab, Jurkat cells were treated with these antioxidants for 2 h. The effects of these inhibitors on tumor-induced apoptosis of lymphocytes were assessed by the JAM assay. As shown in Fig. 6*A*, loss in ^3H-labeled DNA in Jurkat cells coincubated with tumor cells was significantly reduced in the presence of either one of these antioxidants. However, apoptosis induced by agonistic anti-Fas Ab was significantly less affected. These results suggest that tumor-induced apoptosis of T cells involves the generation of ROS and is significantly inhibited by specific antioxidants.

Next, the effects of two mitochondria-specific inhibitors, BA and CsA, were examined. BA, a specific inhibitor of PT and a ligand of ANT in the inner mitochondrial membrane, can inhibit the preapoptotic $\Delta\psi m$ disruption (27, 28). CsA prevents mitochondrial PT by

[5] B. R. Gastman and H. Rabinowich, unpublished data.

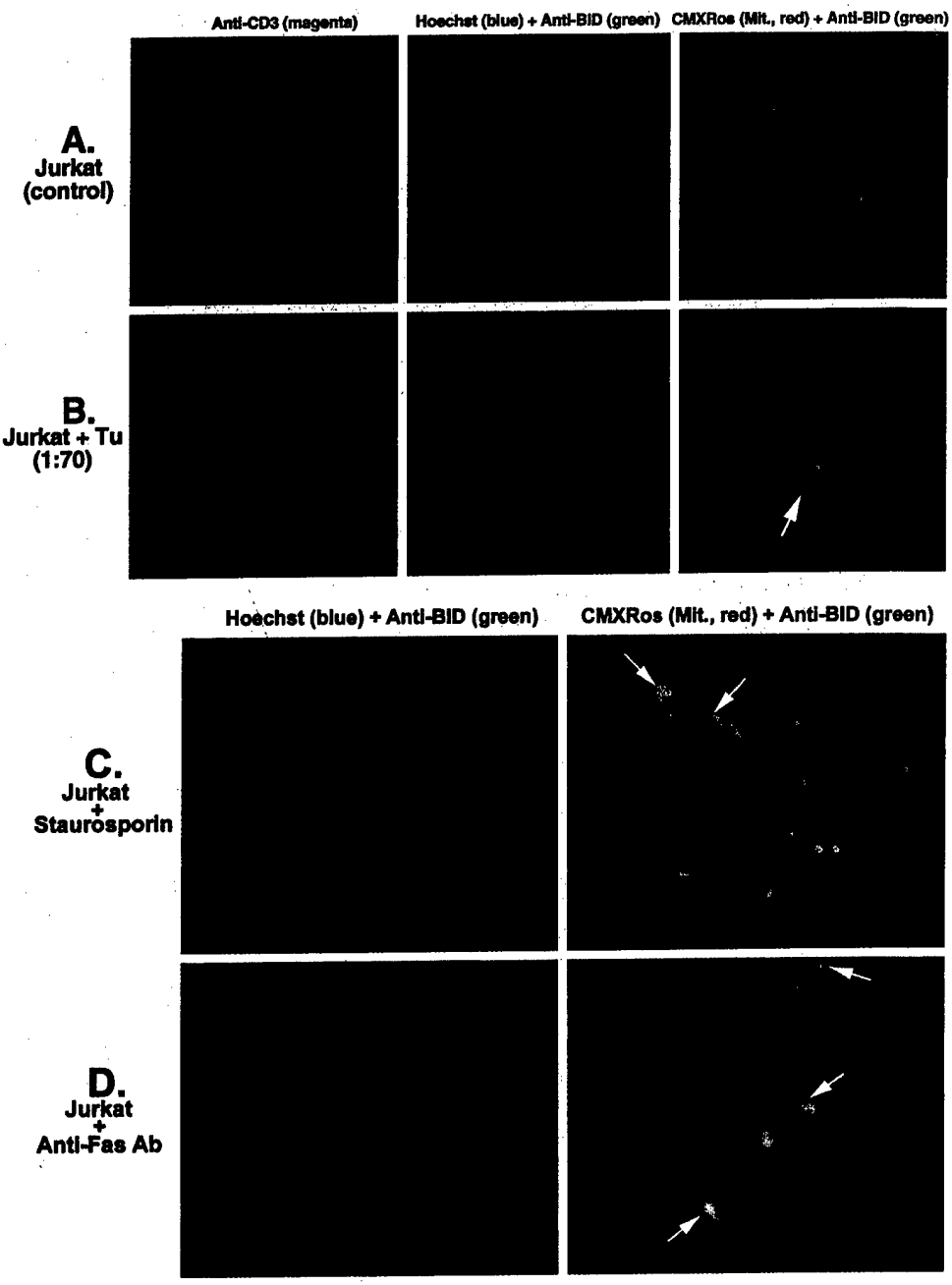

Fig. 3. Immunofluorescence of BID visualized with FITC-conjugated secondary Ab (*green*) in colocalization with CMXRos-stained mitochondria (*red/orange*) in apoptotic Jurkat cells. The different panels include control untreated Jurkat cells (*A*), Jurkat cells cocultured with tumor cells (*B*; tumor:lymphocyte cell ratio, 70:1; 16 h), and Jurkat cells treated with staurosporin (*C*; 0.5 μM, 16 h) or with anti-Fas Ab (*D*; 200 ng/ml, 16 h). Each of the panels demonstrates one cytospin visualized by different stainings. In untreated cells (*A*), BID (*green*) and mitochondria (*red/orange*) are found in the cytoplasm with no evidence for colocalization. In apoptotic Jurkat cells, BID is detected in the cytoplasm showing substantial overlap with CMXRos-stained mitochondria. Colocalization of mitochondria (*red/orange*) and BID (*green*) results in a *yellow* color detected in apoptotic T cells indicated by *arrows* in *B–D* (*right panels*). In *B*, staining with anti-CD3 (*magenta*) served to identify T cells among the cocultured tumor cells.

blocking translocation of mitochondria matrix-specific cyclophilin D to the mitochondria inner membrane, thereby decreasing the sensitivity of mitochondrial megachannels to calcium ions (29). As shown in Fig. 6*B*, these inhibitors significantly ($P < 0.01$, Mann-Whitney U test) blocked mitochondria-dependent apoptosis of Jurkat cells induced by etoposide or diamide but had no effect on death induced by agonistic anti-Fas Ab tested at various concentrations. However, a significant inhibitory effect of either BA or CsA was detected on the level of tumor-induced apoptosis. These results further indicate that tumor-induced apoptosis of T cells has a component that is mitochondria dependent, in contrast to the apoptotic cascade induced by direct ligation of surface Fas.

To examine the significance of caspase-9 activation in the stimulated Jurkat cells, the cell-permeable peptide inhibitor of caspase-9, Ac-LEHD.CHO, was used. This reversible inhibitor had no effect on apoptosis induced by anti-Fas Ab but significantly inhibited the apoptotic effects of VP-16 or tumor cells (Fig. 6*C*). Because caspase-9-dependent VP-16-induced cell death was significantly blocked by Ac-LEHD.CHO, this inhibitor appears to mainly target the mitochondrial pathway of apoptosis. Thus, the partial inhibition of tumor-induced apoptosis of Jurkat cells by this peptide also suggests that the apoptotic process observed is partly mitochondria dependent.

Discussion

In the current study, we demonstrate that mitochondria are involved in apoptotic cascades induced in T cells by either Fas ligation or SCCHN. However, these two cascades are differentially susceptible to a panel of inhibitors of mitochondrial apoptotic events. Whereas Fas-mediated apoptosis in Jurkat cells is executed in the presence of mitochondria-specific inhibitors, tumor-induced apoptosis is partially

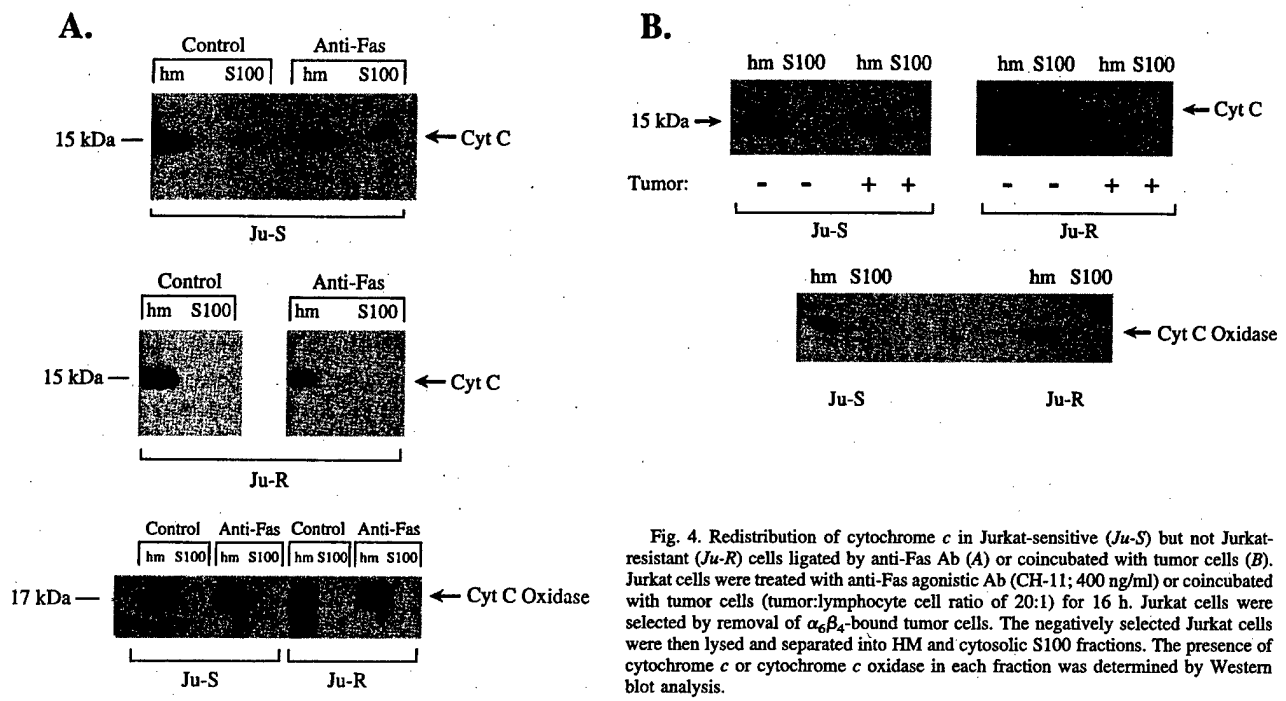

Fig. 4. Redistribution of cytochrome *c* in Jurkat-sensitive (*Ju-S*) but not Jurkat-resistant (*Ju-R*) cells ligated by anti-Fas Ab (*A*) or coincubated with tumor cells (*B*). Jurkat cells were treated with anti-Fas agonistic Ab (CH-11; 400 ng/ml) or coincubated with tumor cells (tumor:lymphocyte cell ratio of 20:1) for 16 h. Jurkat cells were selected by removal of $\alpha_6\beta_4$-bound tumor cells. The negatively selected Jurkat cells were then lysed and separated into HM and cytosolic S100 fractions. The presence of cytochrome *c* or cytochrome *c* oxidase in each fraction was determined by Western blot analysis.

inhibited, suggesting that it is significantly amplified by a mitochondrial cascade.

Two main pathways of caspase activation have been delineated (30). In the first pathway, activation of initiator caspase-8 or caspase-10 is triggered by ligation of death receptors, including Fas, TNFR1, or Death Receptor 3. The second pathway is essentially controlled by mitochondria. Induction of cell death in response to a variety of apoptotic stimuli is associated with mitochondrial release of cytochrome *c*, an event that is blocked by antiapoptotic members of the Bcl-2 family and promoted by proapoptotic members, such as Bax and Bak (12, 13, 31). In the cytosol, cytochrome *c*, together with dATP, forms a complex with APAF-1 that results in activation of caspase-9 and downstream caspases (9). In chemical- or irradiation-induced apoptosis, cytochrome *c* release appears to be caspase independent because it is not inhibited by the pan-caspase inhibitor Z-VAD.FMK (12, 13, 32). Potential mechanisms for the release of cytochrome *c* include opening of mitochondrial PT pores, the presence of specific channels for cytochrome *c* release, or mitochondrial swelling and rupture of the outer membrane, but without loss of mitochondrial membrane potential (33). Because mitochondria-dependent events were also induced by cross-linking of Fas, this organelle has been proposed to act as an amplifier of death receptor signaling (30, 34). Recent studies have highlighted the role of BID, a BH3 domain-containing proapoptotic Bcl-2 family member, in cytochrome *c* release (16, 20). On ligation of death receptors and caspase-8 activation, BID is cleaved and translocates to the mitochondria, where it induces the release of cytochrome *c*.

We and others have recently reported that solid tumors induce the Fas apoptotic pathway in interacting T lymphocytes (3, 4, 18, 19, 35–37). This Fas-mediated cell death may be induced directly by Fas ligand expressed on tumor cells (3) or by activation-induced cell death mediated by up-regulation of receptors and/or ligands of the Fas- or tumor necrosis factor-related apoptosis-inducing ligand pathways in T lymphocytes (38, 39). To date, tumor-induced apoptosis of T cells has only been implicated with death receptor pathways of apoptosis (40–42). In the current study, apoptosis-associated alterations in mitochondria served to confirm the involvement of mitochondria in the signaling phase of tumor-induced apoptosis, and a variety of inhibitors specific for different mitochondrial effector molecules served to reaffirm the significance of this amplification loop. The inhibitors included in this analysis targeted the generation of ROS, changes in mitochondrial transmembrane potential, or activity of caspase-9.

Mitochondria are the major source of oxidants, which are generated as a result of a decrease in coupling efficiency during electron chain transport (24). Generation of ROS is increased during apoptosis induced by a myriad of stimuli (43), suggesting that intracellular oxidation may be a general feature of the mitochondrial effector phase of apoptosis. Our results, which demonstrate an effective inhibition of tumor-induced apoptosis by antioxidants, suggest that in contrast to anti-Fas-mediated apoptosis, mitochondria are actively involved in tumor-induced T-cell death.

During the effector phase of mitochondria-dependent apoptosis, the inner transmembrane potential of the mitochondria collapses (44), indicating the opening of large conductance channels known as mitochondrial PT pores. The structure and composition of the PT pore, which is only partially defined, includes both inner membrane proteins, such as ANT, and outer membrane proteins, such as the voltage-dependent anion channel. The inner and outer membrane proteins operate in concert to create the conductance channels (34). Inhibitors

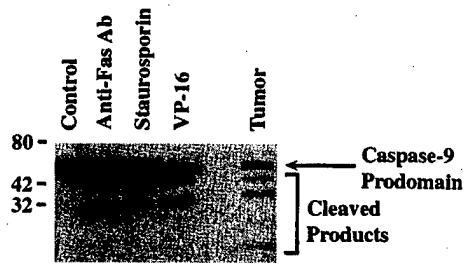

Fig. 5. Activation of caspase-9 in Jurkat cells triggered by anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), VP-16 (20 μM), or cocultured with tumor cells. After coculture, Jurkat cells were negatively selected by removal of tumor cells as described in "Materials and Methods." Immunoblot analysis of whole cell extracts of Jurkat cells was performed as described above. The presence of caspase-9 prodomain or cleaved products was assessed by probing with rabbit anti-caspase-9 polyclonal Ab (Santa Cruz Biotechnology).

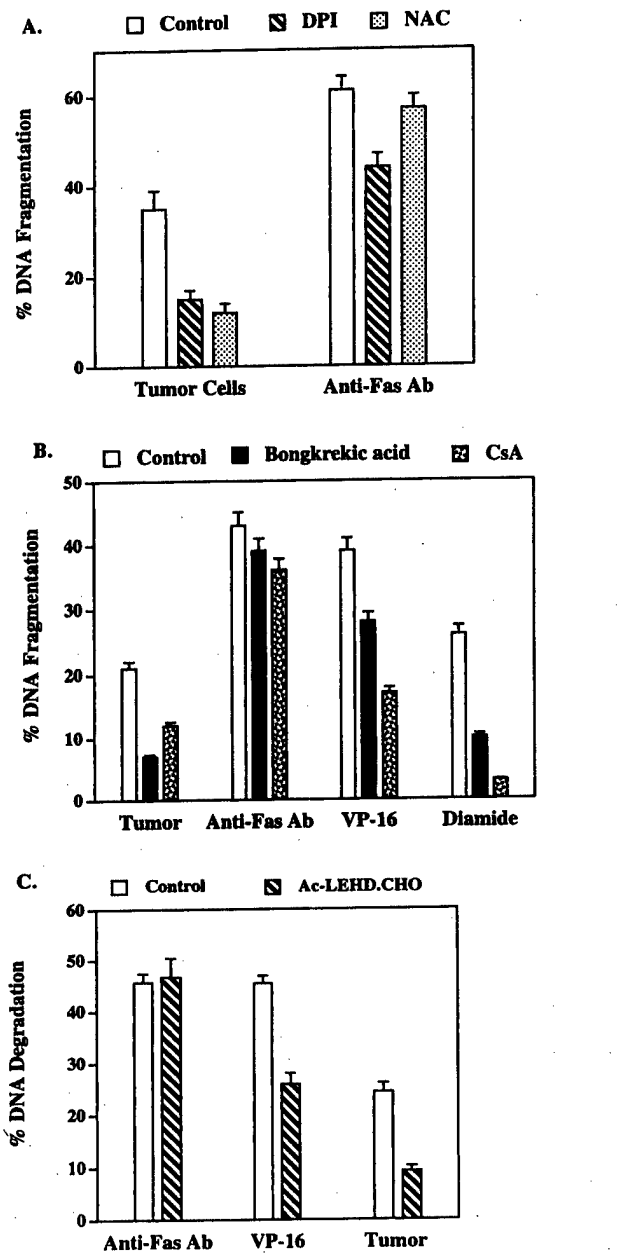

Fig. 6. *A*, inhibition of tumor-induced apoptosis in lymphocytes by antioxidants. [^{3}H]dThd-labeled Jurkat cells were treated with DPI (25 μM) or NAC (10 mM) before coincubation with tumor cells. After coincubation with tumor cells (tumor:lymphocyte cell ratio of 40:1), loss of ^{3}H-labeled DNA was assessed by the JAM assay. The *error bars* represent the SE of eight replicates. *B*, inhibition of tumor-induced but not Fas-induced apoptosis by BA or CsA, inhibitors of PT. Jurkat cells were pretreated with BA (50 μM) or CsA (25 μM) 2 h before the addition of tumor cells (tumor:lymphocyte cell ratio of 40:1) or agonistic anti-Fas Ab (CH-11; 200 ng/ml), VP-16 (20 μM), or diamide (200 μM) for 16 h. The level of apoptosis was assessed by the JAM assay. *C*, effects of caspase-9 inhibitors on Fas-, VP-16-, or tumor-induced apoptosis of Jurkat cells. Jurkat cells were incubated for 2 h with the caspase-9 inhibitor Ac-LEHD.CHO at 25 μM before the addition of an apoptotic stimulus. [^{3}H]dThd-labeled Jurkat cells were incubated in the presence of 200 ng/ml anti-Fas Ab (CH-11), VP-16 (20 μM), or tumor cells (tumor: lymphocyte cell ratio of 50:1) for 16 h. Loss of ^{3}H-labeled DNA was assessed by the JAM assay. The *error bars* represent the SE of eight replicates. Each of the panels represents results obtained in at least three independent experiments.

of the PT pore opening, including CsA, which binds cyclophilin D (associated with ANT), and BA, which also binds ANT, block the PT pore formation. Because these pharmacological inhibitors of the PT pore did not inhibit apoptosis induced by agonistic anti-Fas Ab but did inhibit the mitochondrial cascade initiated by VP-16, diamide, or tumor cells, the phase of PT pore formation appears to be central in the affected apoptotic pathways.

Caspase-9 knockout mice are resistant to apoptotic signals that stimulate the mitochondrial pathway (45), suggesting that caspase-9 plays a central role in mitochondria-dependent pathways of apoptosis. The presence of cleaved products of caspase-9 in tumor-induced apoptotic T cells and the inhibition of death by a caspase-9-specific inhibitor further demonstrate that a mitochondrial cascade plays a significant role in tumor-induced apoptosis of the Jurkat T cells.

BID cleavage and translocation to the mitochondria suggest that the observed mitochondria-dependent events induced in T lymphocytes by tumor cells are, at least in part, related to activation of caspase-8 by death receptors. In such a case, cross-communication between caspase-8 and caspase-9 would be related to the same triggering event and would serve to increase the efficiency of death induced by interaction with tumor cells. It would therefore be expected that inhibition of the mitochondrial amplification loop of caspase activation would attenuate a cascade initiated by death receptors. Interestingly, the various inhibitors of the mitochondrial effector phase of apoptosis used had no effect on cell death induced by ligation of Fas on the surface of Jurkat cells. These observations suggest that the mitochondrial amplification of the Fas cascade in Jurkat cells is not significant. However, each of the inhibitors used significantly hindered Jurkat cell death induced by SCCHN cells. These findings suggest that besides the Fas-mediated pathway, additional mitochondria-dependent cascades are involved in apoptosis of tumor-associated lymphocytes. Alternatively, it is possible that the Fas signaling mediated at the tumor microenvironment is weaker than that delivered by direct cross-linking of Fas on Jurkat cells by agonistic anti-Fas Ab. In the case of insufficient signal, a tumor-initiated caspase-8 cascade would be dependent on cleavage of BID and subsequent mitochondrial amplification of the apoptotic cascade. In any event, tumor-induced apoptosis of Jurkat cells appears to be significantly attenuated by inhibitors that specifically target mitochondrial effector molecules.

Scaffidi *et al.* (15) characterized the Jurkat cells used in their studies as type II, *i.e.*, dependent on mitochondria for execution of Fas signaling, because in those cells the formation of the death–inducing signaling complex was not efficient. Although derived from the same source (46), uncloned Jurkat cell lines propagated for years by different groups are composed of variable mixtures of T-cell populations. Indeed, lines and clones of Jurkat cells resistant to Fas, tumor necrosis factor-related apoptosis-inducing ligand, or other apoptotic stimuli have been selected from apoptosis-sensitive Jurkat cell lines (18, 47, 48). In contrast to the Jurkat cell line used by Scaffidi *et al.* (15), the Jurkat cell line used in the current study was mitochondria independent for execution of apoptosis stimulated by the agonistic anti-Fas Ab. Similar characterization of Jurkat cells as mitochondria independent for Fas signaling has also been reported by others (49). Our findings of differential requirements for mitochondria in the execution of apoptosis of the same cell type suggests that the effector role of mitochondria is stimulus dependent.

In summary, the present study demonstrates that a mitochondrial cascade is contributing to the apoptotic mechanism induced in T cells by SCCHN. Blocking of this apoptotic loop may be important for the success of T-cell-based immunotherapeutic regimens in cancer.

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Advances in Brief

Inhibitor of Apoptosis Protein hILP Undergoes Caspase-mediated Cleavage during T Lymphocyte Apoptosis[1]

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Abstract

Several endogenous or viral inhibitors of apoptosis, including Bcl-2, Bcl-x_L, FLIP, p35, and CrmA, have been shown to be cleaved by caspases during apoptosis. In this study, we demonstrate that the endogenous inhibitor of apoptosis, hILP/XIAP, is also cleaved in apoptotic T lymphocytes, generating at least one prominent fragment of 29 kDa. This p29 cleaved fragment was detected in Jurkat cells induced to apoptose by anti-Fas antibody, staurosporin, or VP-16. The cleavage of hILP appears to be caspase mediated because the production of the p29 protein was inhibited by the pan-caspase peptide inhibitor, Z-VAD.FMK. In Jurkat cells engineered to overexpress CrmA, cleavage of hILP in response to anti-Fas antibody or staurosporin was inhibited, whereas overexpression of Bcl-2 abrogated the cleavage in response to VP-16. Cleavage of hILP was also observed in cell-free reactions using *in vitro* translated hILP and recombinant caspase-3 or -7. Moreover, we found that the p29 hILP fragment retained the ability to bind caspase-3 and -7, as shown previously for full-length or BIR-2 hILP. The p29 cleavage product was also detected during T-cell receptor-mediated apoptosis in peripheral blood lymphocytes from normal donors. Furthermore, tumor-associated T lymphocytes purified from ascites of patients with ovarian cancer expressed fragmented hILP, which was not detected in control T cells purified from peripheral blood of normal donors. Our results suggest that the cleavage of hILP represents an important event in apoptosis of T lymphocytes in both normal and pathological *in vivo* settings.

Introduction

The execution of cellular apoptosis involves the activation of a cascade of intracellular proteases belonging to the caspase protease family (1, 2). Caspases are initially synthesized as inactive proenzymes, and activation involves processing to smaller active subunits. Activation of the apical proteases caspase-8 (3–7) after engagement of cell surface death receptors or caspase-9 (8, 9) after release of cytochrome *c* from mitochondria results in processing and activation of downstream executioner caspases including caspase-3 (8, 10). Executioner caspases cleave specific cellular substrate proteins, facilitating the demise of the cell (1, 2).

A number of intracellular proteins that negatively regulate apoptosis execution, primarily by interfering with the caspase cascade, have been identified. Antiapoptotic members of the Bcl-2 protein family act to prevent release of cytochrome *c* from the mitochondria (11, 12) and can also bind and incapacitate Apaf-1 (13, 14), a critical cytoplasmic protein involved in cytochrome *c*-mediated activation of caspase-9 (8, 15). c-FLIP, a death effector domain-containing protein, prevents association of caspase-8 with cell surface death receptors, thereby blocking caspase-8 activation (16). More recently, it has been shown that members of the IAP (17) protein family bind and inhibit specific caspases (18–20).

Human IAP proteins, including hILP/XIAP (21, 22), c-IAP1, c-IAP2, NAIP, survivin, and Bruce, are characterized by the presence of one to three copies of a 70-amino acid motif, the BIR domain, which bears homology to sequences found in the baculovirus IAP proteins (reviewed in Ref. 17). The hILP, c-IAP1, and c-IAP2 proteins also contain COOH-terminal RING finger domains. hILP, c-IAP1, and c-IAP2 bind and inhibit the activated forms but not the proenzyme forms of caspase-3 and -7 (18–20). In addition, hILP, c-IAP1, and c-IAP2 bind procaspase-9, preventing processing and activation of this enzyme (18). These inhibitors also inhibit active caspase-9. However, despite demonstrations that hILP, c-IAP1, and c-IAP2 can bind and inhibit caspases, the molecular mechanism(s) of this inhibition remains unclear. Two caspase inhibitor proteins that are unrelated to IAPs, cowpox viral CrmA (23) and baculovirus p35 (24, 25), also bind directly to caspases (25). CrmA and p35 have been shown to be suicide inactivators of caspases (26–28). After binding, CrmA and p35 are proteolytically cleaved, and the cleaved products remain associated with the caspase to inhibit enzyme activity.

The hILP protein consists of three BIR domains and one RING finger domain and appears to be a more potent inhibitor of caspase-3 and -7 than c-IAP1 or c-IAP2 (19, 20). The RING finger domain is not essential for hILP binding to caspase-3 and -7, but it is important for binding to the cytoplasmic domain of bone morphogenetic protein type I receptor and may mediate functions of hILP unrelated to apoptosis (29). Of the three hILP BIR domains, the second BIR domain, but not the first or third domains, is sufficient for binding and inhibition of caspase-3 and -7 (30). Thus, only a portion of the molecule is needed for caspase inhibition and suppression of apoptosis.

In this report, we demonstrate that cellular hILP is cleaved by a caspase protease after treatment of cells with a variety of apoptotic stimuli. Similar hILP cleavage was seen *in vitro* using recombinant caspase-3 or -7 and *in vitro* translated hILP as a substrate. The cleavage products were found to remain associated with caspase-3 and -7 and were detected in peripheral blood T cells from healthy individuals stimulated to undergo AICD[3] and detected *in vivo* in TALs. These findings demonstrate that hILP is a substrate of caspases and may act as a suicide inactivator of these enzymes.

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[3] The abbreviations used are: AICD, activation-induced cell death; Ab, antibody; mAb, monoclonal antibody; PBL, peripheral blood lymphocyte; TAL, tumor-associated lymphocyte; PMA, phorbol 12-myristate 13-acetate; PVDF, polyvinylidene difluoride; OvCA, ovarian carcinoma.

Materials and Methods

Reagents. Agonistic anti-Fas Ab (CH-11; IgM) was purchased from Upstate Biotechnology (Lake Placid, NY); Staurosporin, etoposide (VP-16), PMA, and ionomycin were purchased from Sigma (St. Louis, MO). Anti-$\alpha_6\beta_4$ mAb (A9) was a generous gift from Dr. T. E. Carey (University of Michigan, Ann Arbor, MI). Inhibitors of apoptosis, including Z-VAD.FMK and Z-DEVD.FMK, were purchased from Enzyme Systems (Livermore, CA). Recombinant caspase-3 and caspase-7 were purchased from PharMingen (San Diego, CA). A mAb specific for hILP was purchased from Transduction Laboratories (San Diego, CA). Rabbit anti-caspase-3 Ab and murine anti-caspase-7 mAb were purchased from PharMingen. Anti-CD3 mAb was purchased from DAKO (Carpinteria, CA).

Cell Lines. Jurkat T leukemic cell line was obtained from American Type Culture Collection (Manassas, VA). Jurkat cells were grown in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin (complete medium). The generation of stable cell lines expressing epitope-tagged CrmA or Bcl-2 proteins has been described previously (31). Transfected cell lines were maintained in complete medium supplemented with 0.5 mg/ml G418 (Life Technologies, Inc.).

Induction of Apoptosis. Jurkat cells plated at 0.5–1 $\times$ 10^6 cells/ml in complete medium were treated with VP-16 (20 μM), agonistic anti-Fas Ab (200 ng/ml), or staurosporin (0.5 μM) at 37°C for varying lengths of time, as indicated for each experiment. To induce AICD, 48-well plates were precoated with anti-CD3 (5 μg/ml) in 50 mM Tris (pH 9.0). PBL-T cells (1 $\times$ 10^6 cells/ml) were plated in wells precoated with anti-CD3 mAb in complete medium in the presence of PMA and ionomycin at concentrations of 50 ng/ml and 0.5 μg/ml, respectively (32).

Western Blot Analysis. To generate whole cell extracts, cells were lysed in 1% NP40, 20 mM Tris-base (pH 7.4), 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Proteins were resolved by SDS-PAGE using 15% polyacrylamide gels and transferred to PVDF membranes as described previously (33). The protein bands were detected by enhanced chemiluminescence (Pierce, Rockford, IL) after probing with a specific primary Ab and a horseradish peroxidase-conjugated secondary Ab.

***In Vitro* Translation.** Human hILP cDNA cloned in pcDNA3 vector was a generous gift from Colin S. Duckett (NIH, Bethesda, MD). Plasmid DNA was translated in the TNT T7 transcription-translation-coupled reticulocyte lysate system (Promega). Each coupled transcription-translation reaction contained 1 μg of plasmid DNA in a final volume of 50 μl in a methionine-free amino acid mixture supplemented with [^{35}S]methionine according to the manufacturer's instructions. After incubation at 30°C for 90 min, the reaction products were stored at −70°C.

***In Vitro* Cleavage Reaction.** *In vitro* cleavage reactions were performed in a buffer containing 20 mM HEPES (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin for 1 h at 30°C with 4 μl of ^{35}S-labeled hILP protein in the presence or absence of recombinant caspases and peptide inhibitors of caspases in a total volume of 20 μl/reaction. Reactions were terminated by the addition of SDS loading buffer and boiling for 5 min. Products of the cleavage reactions were resolved on 15% SDS-PAGE, transferred to PVDF membranes, and detected by autoradiography. Alternatively, the reaction products were detected by Western blotting with anti-hILP mAb.

Patients. Ascitic fluids were obtained from patients with OvCA at Magee Women's Hospital, University of Pittsburgh Medical Center (Pittsburgh, PA). The ovary was the primary site of malignancy for all patients. The patients were untreated at the time of specimen collection. The study was approved by the Institutional Review Board for human use at the University of Pittsburgh Medical Center.

Isolation of TAL-T or PBL-T Cells. Ascitic fluid cells were washed twice in RPMI 1640, placed on Ficoll-Hypaque discontinuous density gradients, and centrifuged to harvest TAL and tumor cells as described previously (34). To select for T lymphocytes, TALs were incubated in the presence of anti-CD14, anti-CD16, anti-CD19 (10 μg/ml; DAKO) and anti-$\alpha_6\beta_4$ mAbs (50 μg/ml) for 30 min at 0°C. The cells were then washed twice and incubated with magnetic beads coated with goat antimouse immunoglobulins (1 cell:30 beads; PerSeptive Diagnostics, Cambridge, MA) for 30 min at 0°C. After each of two successive incubations with magnetic beads, a magnet was used to collect beads containing attached cells. T cells from peripheral blood on normal donors were purified by a similar procedure. As assessed by flow cytometry, the negatively selected T cells were 99% CD3 positive.

Results and Discussion

Cleavage of hILP in Apoptotic Jurkat Cells. To determine the fate of cellular hILP protein during apoptotic execution, Jurkat T leukemic cells were stimulated with 200 ng/ml agonistic anti-Fas Ab for varying lengths of time. After stimulation, whole cell lysates were prepared and analyzed by immunoblotting using mAb raised against amino acids 268–426 of the human hILP protein. As expected, full-length hILP was detected as a 57-kDa protein (Fig. 1*A*). After 16 h of stimulation with anti-Fas Ab, the level of full-length hILP was

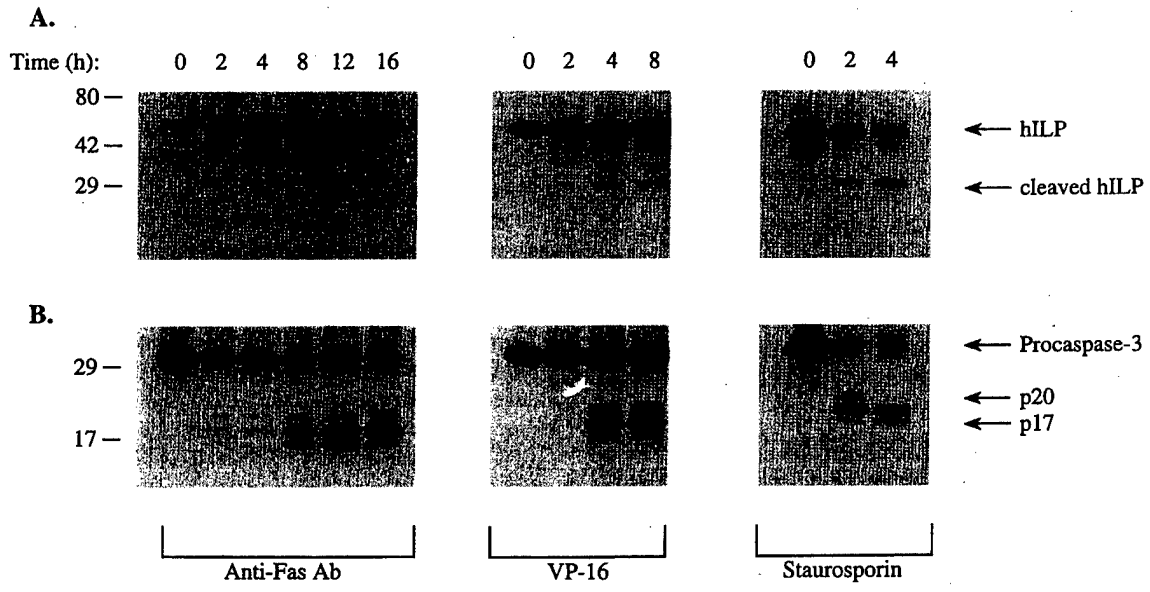

Fig. 1. Cleavage of hILP protein during apoptosis induced by anti-Fas Ab, VP-16, or staurosporin. Jurkat T cells were treated 200 ng/ml agonistic anti-Fas mAb, 20 μM VP-16, or 0.5 μM staurosporin at 37°C for varying lengths of time. After treatment, whole cell lysates were prepared. Proteins (25 μg/lane) were resolved on 15% SDS-PAGE gels and transferred to PVDF membranes. In *A*, the membranes were probed with anti-hILP mAb. In *B*, the membranes were stripped and reprobed with polyclonal anti-caspase-3 Ab.

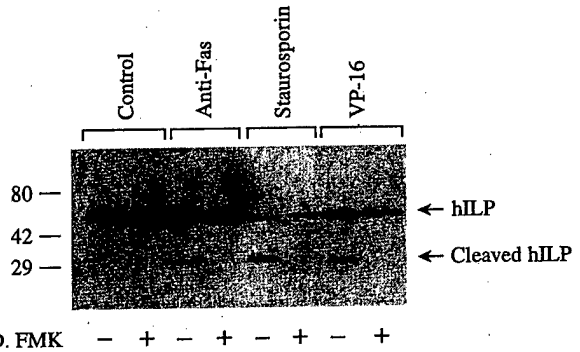

Fig. 2. Inhibition of hILP cleavage by the caspase inhibitor Z-VAD.FMK. Jurkat cells were treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) in the presence or absence of Z-VAD.FMK (50 μM). Whole cell lysates were electrophoresed on 15% SDS gels and analyzed by immunoblotting using anti-hILP mAb.

significantly reduced. Even more apparent was the appearance of a 29-kDa fragment recognized by the anti-hILP mAb. The 29-kDa fragment was first detected after 2 h of stimulation, and its levels continued to increase thereafter. Stimulation of Jurkat cells with anti-Fas Ab also resulted in the processing of procaspase-3 (32 kDa) to active subunits of 20 and 17 kDa (Fig. 1*B*). Activation of caspase-3 was first detected after 2 h of treatment but was far more substantial after 8 h of treatment. Thus, cleavage of hILP occurred concurrently with caspase-3 activation.

Cleavage of hILP to a 29-kDa fragment was also observed after treatment with 20 μM VP-16 or 0.5 μM staurosporin (Fig. 1*A*). The 29-kDa fragment was first detected after 4 h of treatment with VP-16 and after 2 h of treatment with staurosporin. Processing of caspase-3 coincided with production of the hILP fragment.

Inhibition of hILP Cleavage by Z-VAD.FMK. A number of intracellular proteins are cleaved by caspase proteases during apoptosis, including the antiapoptotic molecules Bcl-2 and Bcl-X_L (35–38). In addition, the CrmA and p35 proteins, which directly bind and inhibit caspases, have been shown to be caspase substrates (26–28). Because hILP is known to bind and inhibit caspase-3 and -7, we sought to determine whether hILP cleavage in apoptotic cells was mediated by a caspase protease. To address this question, Jurkat cells were treated with anti-Fas, staurosporin, or VP-16 in the absence or presence of Z-VAD.FMK, a potent general inhibitor of caspases. As shown in Fig. 2*A*, Z-VAD.FMK completely abrogated production of the 29-kDa-hILP fragment in response to all three stimuli. This indicated that hILP cleavage was caspase mediated.

Inhibition of hILP Cleavage by Bcl-2 and CrmA. To further examine the involvement of caspases in hILP cleavage, we studied the effects of two inhibitors of caspase activation, Bcl-2 and CrmA. Bcl-2 inhibits caspase activation by blocking release of cytochrome *c* from the mitochondria and may also interact with Apaf-1, disrupting caspase-9 activation (11–14). Because Bcl-2 blocks cytochrome *c* release, it is a potent inhibitor of stimuli that primarily use the mitochondrial pathway of apoptosis, such as chemotherapeutic agents. By contrast, Bcl-2 is much less efficient at inhibiting Fas-mediated apoptosis, which is not dependent on the mitochondrial pathway. The CrmA protein binds and potently inhibits caspase-8, the apical caspase in Fas-mediated signaling (25). Thus, CrmA strongly inhibits anti-Fas-induced apoptosis. On the other hand, chemotherapy-induced caspase activation and apoptosis are only modestly inhibited by CrmA.

Jurkat cells engineered to overexpress Bcl-2 or CrmA (31) were treated with anti-Fas Ab, staurosporin, or VP-16, followed by immunoblot analysis with anti-hILP (Fig. 3). As a control, cells transfected with vector alone (*Neo*) were also analyzed. Bcl-2 overexpression completely abrogated hILP cleavage in response to VP-16 treatment. Considerably less protection was seen in Bcl-2/Jurkat cells treated with anti-Fas or staurosporin. CrmA, on the other hand, dramatically inhibited hILP cleavage in the anti-Fas- and staurosporin-treated cells but had little impact in VP-16-treated cells. Taken together, these results support the conclusion that cleavage of hILP in apoptotic cells is mediated by a caspase protease.

Cleavage of hILP by Recombinant Caspases. To identify caspases involved in hILP cleavage, *in vitro* translated ^{35}S-labeled hILP protein was prepared and incubated with active, recombinant caspase-3 or caspase-7. As assessed by autoradiography, a p29 fragment was generated by each of the two caspases (Fig. 4, *A* and *B*). Production of the cleavage fragments was inhibited by the caspase inhibitor Z-DEVD.FMK. It appears that *in vitro* translated hILP is more accessible to recombinant caspases than the endogenous hILP is to endogenous caspases because additional proteolytic fragments were detected *in vitro*. However, like the endogenous fragment, the p29 cleavage fragment generated from *in vitro* translated hILP was detected by anti-hILP mAb (Fig. 4, *C* and *D*).

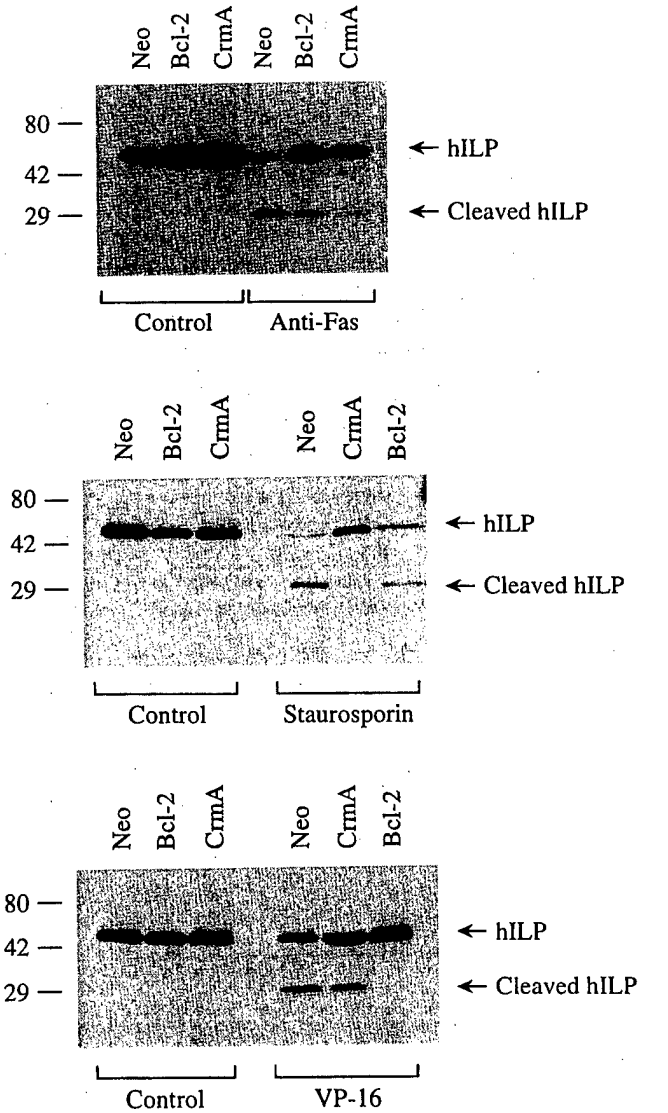

Fig. 3. Inhibition of hILP cleavage by Bcl-2 and CrmA. Clonal Jurkat cell lines engineered to overexpress Bcl-2 or CrmA were treated as described in the Fig. 2 legend. Jurkat cells transfected with vector alone (*Neo*) served as control. After treatment, whole cell extracts were resolved by SDS-PAGE, transferred to PVDF membranes, and probed with anti-hILP mAb.

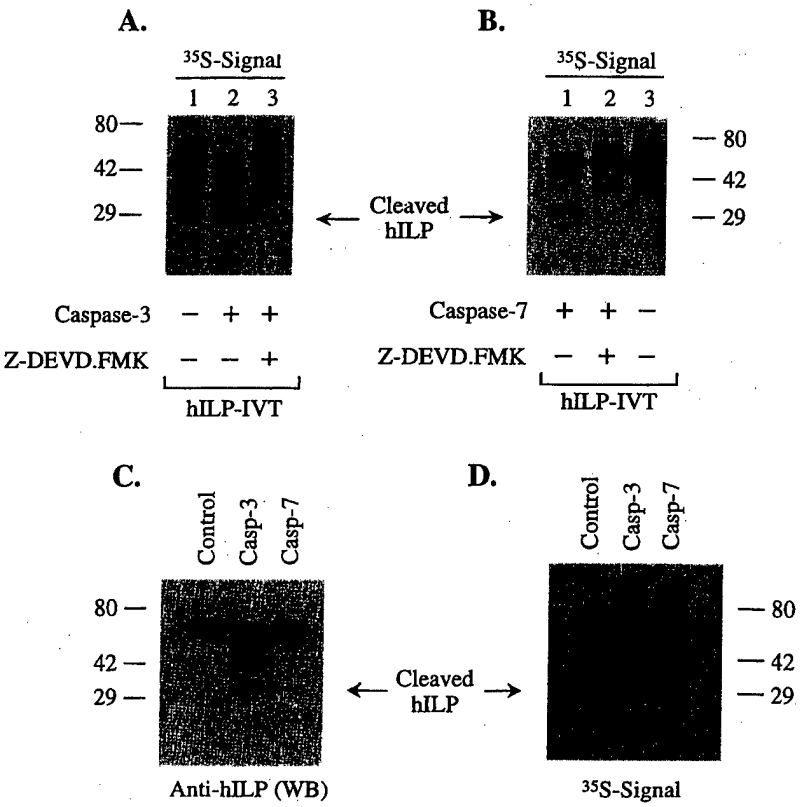

Fig. 4. *In vitro* translated ^{35}S-labeled hILP is cleaved by recombinant caspase-3 or caspase-7. *In vitro* translated ^{35}S-labeled hILP was treated for 1 h with recombinant caspase-3 (*A*) or caspase-7 (*B*; 0.2 μg enzyme/reaction) in the presence or absence of Z-DEVD.FMK (50 μM). The reaction products were resolved by SDS-PAGE and transferred to PVDF membranes. The membranes were analyzed by autoradiography (*A*, *B*, and *D*) or by Western blotting with anti-hILP mAb (*C*).

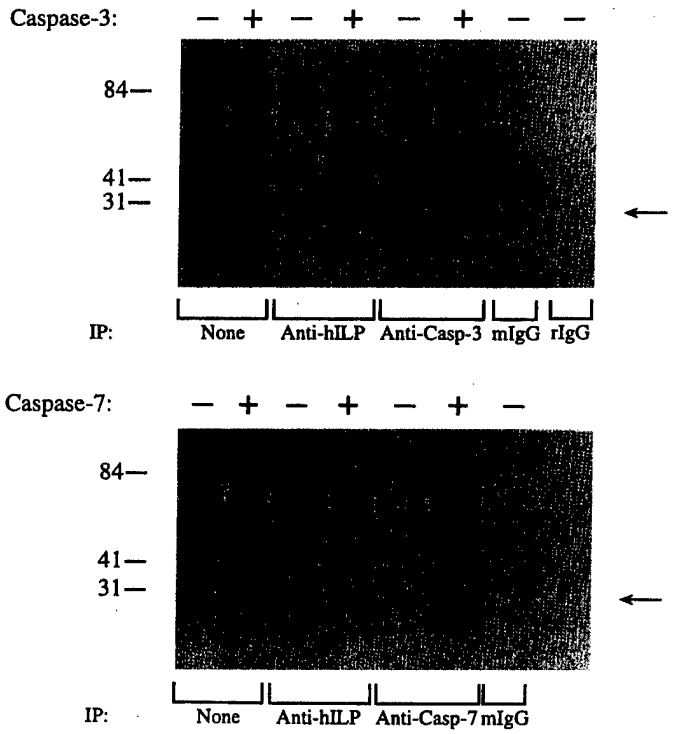

Fig. 5. Association of the p29 cleavage product of ^{35}S-labeled hILP with recombinant active subunits of caspase-3 or caspase-7. *In vitro* translated ^{35}S-labeled hILP was treated for 1 h at 30°C with recombinant caspase-3 (*top*) or recombinant caspase-7 (*bottom*). After treatment, immunoprecipitating Abs specific for hILP, caspase-3, or caspase-7 were added. Immune complexes were selected using either protein G (for anti-hILP or anti-caspase-7 mAb) or protein A (for anti-caspase-3 Ab). The immune complexes were resolved on 15% SDS gels, transferred to PVDF membranes, and examined by autoradiography. As controls, the *in vitro*-translated ^{35}S-labeled hILP was subjected to no immunoprecipitation or immunoprecipitated with anti-hILP, mouse IgG, or rabbit IgG. The *arrow* indicates the p29 cleavage product immunoprecipitated by anti-hILP or coimmunoprecipitated with either anti-caspase-3 or anti-caspase-7.

Because hILP has been reported to bind caspase-3 and caspase-7 (20, 30), we examined whether the p29 fragment maintains its ability to physically associate with caspases. To this end, *in vitro* translated ^{35}S-labeled hILP was treated with recombinant caspase-3 or caspase-7. The caspases were then immunoprecipitated by a specific anti-caspase Ab and resolved on SDS gels. The ^{35}S-labeled coimmunoprecipitated proteins were examined by autoradiography. As shown in Fig. 5, the p29 fragment was not only immunoprecipitated by anti-hILP Ab but was also coimmunoprecipitated with either caspase-3 or caspase-7. These results demonstrate that although hILP is cleaved by caspases, the p29 product remains associated with the cleaving enzyme. Thus, hILP may function in a fashion similar to that of CrmA and p35, with the cleaved products remaining bound to the enzyme and inhibiting the caspase activity. Alternatively, hILP may behave like Bcl-2, losing its antiapoptotic activity after cleavage and perhaps even becoming proapoptotic (37).

Cleavage of hILP in T-cell Receptor-mediated Apoptosis. To investigate the relevance of hILP cleavage in normal T lymphocytes, peripheral blood T cells from healthy individuals were stimulated to undergo AICD by incubation with immobilized anti-CD3 mAb in the presence of PMA and ionomycin for 14 h at 37°C (32). As assessed by Western blot analyses performed on whole cell extracts, the p29 fragment was detected in T cells activated via the T-cell receptor (Fig. 6*A*, *Lane 1*), but not in control cells stimulated with only PMA and ionomycin (Fig. 6*A*, *Lane 4*). Interestingly, in human peripheral blood T cells, an additional p45 hILP protein was detected in cells treated with anti-CD3, VP-16, or staurosporin, suggesting that hILP may be subjected to cleavage before production of the p29 fragment. In T lymphocytes pretreated with the pan-caspase inhibitor Z-VAD.FMK before stimulation of AICD, no cleavage products of hILP were detected (data not shown).

Detection of p29 hILP in OvCA Ascitic TALs. To further investigate the physiological significance of hILP cleavage, we examined T cells purified from either OvCA ascitic TALs or T cells from periph-

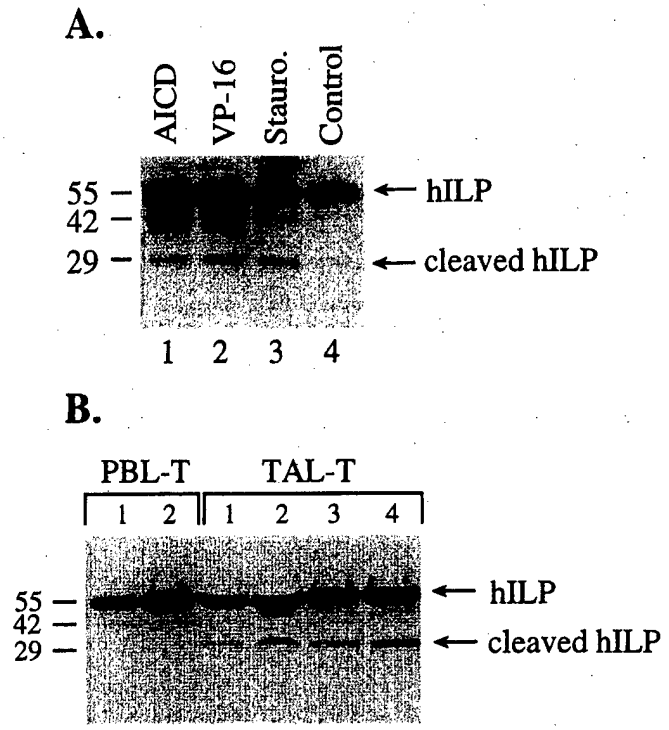

Fig. 6. Detection of the p29 fragment of hILP in PBL-T cells stimulated to undergo AICD (*A*) and in OvCA ascitic TAL-T cells (*B*). In *A*, peripheral blood T cells from a normal donor were stimulated by immobilized anti-CD3 mAb (5 μg/ml), PMA (50 ng/ml), and ionomycin (0.5 μg/ml) to induce AICD (*Lane 1*). PBL-T cells stimulated by PMA and ionomycin served as a negative control (*Lane 4*), and cells stimulated with VP-16 (*Lane 2*; 20 μM) or staurosporin (*Lane 3*; 0.5 μM) served as positive controls. Similar results were obtained with PBL-T cells from three normal individuals. In *B*, TAL-T cells from four OvCA ascites (1–4) and control PBL-T cells from two normal donors (1–2) were purified by negative selection as described in "Materials and Methods." Cell lysates were resolved by 15% SDS-PAGE, transferred to PVDF membranes, and probed with hILP-specific mAb.

eral blood of normal donors. The OvCA TAL population contained a substantial percentage of apoptotic cells (up to 30% terminal deoxynucleotidyl transferase-mediated nick end labeling-positive T cells; Ref. 34). Whole cell extracts were prepared from TAL-T or PBL-T and examined by Western blot analysis for the presence of cleaved products of hILP. The p29 cleaved product of hILP was detected in TAL-T cells prepared from the ascites of four OvCA patients but not in PBL-T cells from two normal donors (Fig. 6*B*). These results demonstrate that the cleavage of hILP occurs in an *in vivo* setting where the loss or gain of inhibitory function may have significant biological consequences.

In summary, we have shown that endogenous hILP is cleaved by a caspase protease during cellular apoptosis. The fact that hILP cleavage is seen after Fas stimulation or treatment with VP-16 or staurosporin indicates that the caspase responsible is active in both death receptor- and drug-mediated apoptotic pathways. The observation that the hILP cleavage products remain associated with active subunits of caspase-3 and -7 suggests that hILP, like CrmA and p35, may act as a suicide inactivator of caspases, undergoing cleavage as a part of its mechanism. However, it is also possible that the cleavage products exhibit some unique cellular function. In this regard, it is interesting to note that sequences in the RING finger domain of hILP promote association of hILP with the cytoplasmic domain of bone morphogenetic protein type I receptor (29). If the hILP cleavage products dissociate from caspases inside the cell, then it is likely that they may act as dominant-negative inhibitors of normal hILP function. Future studies will be needed to thoroughly investigate these possibilities. Our observations that hILP cleavage products are found in peripheral blood T cells undergoing AICD and also in TALs demonstrate that hILP proteolysis occurs *in vivo*. Thus, hILP cleavage may be fundamentally important to the process of apoptosis in both normal and pathological *in vivo* settings.

Note Added in Proof: While this manuscript was under review, a similar pattern of caspase-mediated hILP/XIAP cleavage was reported by Deveraux *et al.* (Q. L. Devereaux *et al.*, EMBO J., *18:* 5242–5251, 1999).

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Apoptosis-resistant Mitochondria in T Cells Selected for Resistance to Fas Signaling*

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Jurkat leukemic T cells are highly sensitive to the extrinsic pathways of apoptosis induced via the death receptor Fas or tumor necrosis factor-related apoptosis-inducing ligand as well as to the intrinsic/mitochondrial pathways of death induced by VP-16 or staurosporin. We report here that clonal Jurkat cell lines selected for resistance to Fas-induced apoptosis were cross-resistant to VP-16 or staurosporin. Each of the apoptotic pathways was blocked at an apical phase, where common regulators of apoptosis have not yet been defined. The Fas pathway was blocked at the level of caspase-8, whereas the intrinsic pathway was blocked at the mitochondria. No processing or activity of caspases was detected in resistant cells in response to either Fas-cross-linking or VP-16 treatment. Also, no apoptosis-associated alterations in the mitochondrial inner membrane, outer membrane, or matrix were detected in resistant Jurkat cells treated with VP-16. Thus, no changes in permeability transition, loss in inner membrane cardiolipin, generation of reactive oxygen species, or release of cytochrome *c* were observed in resistant cells treated with VP-16. Further, unlike purified mitochondria from wild type cells, those obtained from resistant cells did not release cytochrome *c* or apoptosis-inducing factor in response to recombinant Bax or truncated Bid. These results identify a defect in mitochondria ability to release intermembrane proteins in response to Bid or Bax as a mechanism of resistance to chemotherapeutic drugs. Further, the selection of VP-16-resistant mitochondria via elimination of Fas-susceptible cells may suggest the existence of a shared regulatory component between the extrinsic and intrinsic pathways of apoptosis.

Susceptibility to apoptosis is an essential prerequisite for successful treatment of tumor cells by cytotoxic T lymphocytes, natural killer cells, radiation, or chemotherapy. However, resistance to apoptosis has been established as one of the mechanisms responsible for the failure of therapeutic approaches in many types of cancer, including hematopoietic malignancies. At least two pathways of caspase activation have been delineated, including extrinsically and intrinsically stimulated cascades (1–3). The extrinsic pathway involves apoptosis mediated by cell surface death receptors, such as Fas, tumor necrosis factor receptor, or TRAIL[1] receptor. Fas stimulation results in oligomerization of the receptors and recruitment of the adapter protein Fas-associated death domain (FADD) and caspase-8, forming a death-inducing signaling complex (DISC) (4). Autoactivation of caspase-8 at the DISC is followed by activation of effector caspases, including caspase-3, -6, and -7, which function as downstream effectors of the cell death program. The intrinsic pathway is mediated by diverse apoptotic stimuli, which converge at the mitochondria. Release of cytochrome *c* from the mitochondria to the cytoplasm initiates a caspase cascade. Cytosolic cytochrome *c* binds to apoptosis protease-activating factor 1 (Apaf-1) and procaspase-9, generating a DISC-like complex, "apoptosome" (5, 6). Within the apoptosome, caspase-9 is activated, leading to processing of caspase-3. The two pathways of apoptosis, extrinsic/death receptor and intrinsic/mitochondrial, converge on caspase-3 and subsequently on other proteases and nucleases that drive the terminal events of programmed cell death. The significance of Apaf-1, caspase-9, and caspase-3 for the execution of apoptosis has been confirmed by genetic studies in mice (7–9). Cells derived from Apaf-1, caspase-9, and caspase-3 knockout mice demonstrated defects in response to a variety of apoptotic stimuli. However, T lymphocytes from mice lacking Apaf-1 or caspase-9 were susceptible to apoptosis signaled via Fas or tumor necrosis factor receptors (10, 11). Also, cytochrome *c*-deficient cells were susceptible to death receptor-mediated apoptosis (12). These findings support the concept that the two apoptotic pathways are discrete and function in parallel. However, each of the pathways is amplified by activated components of the "other" pathway. In cases of low initial caspase-8 activation, the direct effect of caspase-8 on caspase-3 is amplified by a mitochondrial loop (1). In this scenario, caspase-8 cleaves off an N-terminal fragment of Bid, allowing the tBid to translocate to the mitochondria and induce cytochrome *c* release (13–15). Also, the intrinsic pathway is amplified by components of the death receptor cascade, as cytostatic drugs have been reported to activate caspase-8 secondarily to mitochon-

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[1] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; AIF, apoptosis-inducing factor; Apaf-1, apoptosis protease-activating factor 1; BAR, bifunctional apoptosis regulator; CMXRos, chloromethyl X-rosamine; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FLIP, FLICE-inhibitory protein; HE, hydroethidine/hydroethidium; HM, heavy membrane; NAO, nonyl acridine orange; PARP, poly(ADP-ribose) polymerase; tBid, truncated Bid; VDAC, voltage-dependent anion channel(s); XIAP, X-linked inhibitor of apoptosis; Ab, antibody; mAb, monoclonal antibody; MIB, mitochondria buffer; MOPS, 4-morpholinepropanesulfonic acid.

drial damage (16, 17). Currently, cross-talk between the extrinsic and intrinsic pathways has been observed mainly as an amplification loop at the level of execution, but not initiation, of each of the cascades. Also, tumor cell lines studied for resistance to apoptosis were resistant to either the death receptor or the mitochondrial pathway but not cross-resistant to both pathways (18). Cross-resistance has been observed in cells overexpressing an inhibitor, such as X-linked inhibitor of apoptosis (XIAP/hILP), which targets downstream effector molecules, such as caspase-3, shared by both pathways (19–22). Also, members of the Bcl-2 family, which play a major role in control of the mitochondria-dependent apoptotic pathway (23), were reported to protect some cell lines and tissues from Fas-induced apoptosis but not others (24, 25). However, attenuation of Fas-mediated apoptosis by Bcl-2 might be mediated by inhibition of the mitochondrial amplification loop (1).

In the current study, we demonstrate the selection of cells resistant to a mitochondrial pathway (intrinsic) by elimination of Fas-sensitive cells (extrinsic pathway). The selection of clonal cell lines completely cross-resistant to inducers of the two apoptotic pathways may suggest the existence of an upstream regulatory component shared by the extrinsic and the intrinsic pathways.

EXPERIMENTAL PROCEDURES

Reagents—Agonistic anti-Fas Ab (CH-11, IgM) was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Staurosporin and VP-16 were purchased from Sigma. Recombinant caspase-3 and caspase-8, anti-cytochrome *c* mAb, rabbit anti-caspase-3 Ab, and anti-CD3 ζ-chain mAb (clone 610B10) were purchased from Pharmingen. Anti-hILP/XIAP mAb (clone 28), anti-caspase-7 mAb (clone 51), and anti-caspase-2 mAb (clone 47) were from Transduction Laboratories (Lexington, KY); rabbit anti-caspase-8 Ab was from StressGen Biotechnology (Victoria BC, Canada); caspase-8-specific mAb (clone 5F7) was from Upstate Biotechnology, Inc.; anti-caspase-9 was from Oncogene (Cambridge, MA); anti-Bcl-2 mAb (clone 100), rabbit anti-Bcl-xL, and goat anti-apoptosis-inducing factor (AIF) Ab were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-poly(ADP-ribose) polymerase (PARP) mAb (clone C2–10) was from Enzyme Systems (Livermore, CA); and anti-voltage-dependent anion channel (VDAC) mAb (clone 31HC) was from Calbiochem. Rabbit anti-Bid Ab was generated as described previously (26). Chloromethyl X-rosamine (CMXRos), nonyl acridine orange (NAO), and hydroethidine (HE) were from Molecular Probes, Inc. (Eugene, OR); phosphatidylethanolamine-conjugated anti-APO2.7, fluorescein isothiocyanate-annexin V and propidium iodide were from CLONTECH (Palo Alto, CA); anti-β-actin mAb (clone AC-15), horse heart cytochrome *c*, and dATP were from Sigma; and the TRAIL kit was from Alexis (San Diego, CA).

Preparation of GST-Bax and His-tagged tBid—Mouse Bax-ΔTM (amino acids 1–173) was cloned into pGEX-2T (Amersham Pharmacia Biotech) in fusion with GST protein at its N terminus. Bacterial *Escherichia coli* strain DH5α (Life Technologies, Inc.) was transformed and cultured in LB medium. When A_{600} reached 0.7–1.0, isopropyl β-D-thiogalactoside was added at 0.1 mM to induce the expression of fusion protein. Bacteria were harvested after 2–3 h of incubation at 37 °C and lysed by sonication in lysis buffer (1% Triton X-100, 1 mM EDTA in PBS). After centrifugation, the supernatant was incubated with preswollen glutathione beads for 30 min at 4 °C. The beads were then precipitated and washed by centrifugation. GST-BAX-ΔTM was eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0.

Mouse tBid (amino acids 60–195) was cloned into a pET23dwHis vector modified from the pET23d(+) vector (Novagen, WI), in fusion with the 6-histidine tag at its N terminus. Bacteria *E. coli* strain BL21(DE3) was transformed and cultured at 37 °C in Terrific Broth. The induction of expression was started at 0.8–1.0 A_{600} by the addition of 0.4 mM isopropyl β-D-thiogalactoside at 37 °C for 2–3 h. The bacterial pellets were resuspended and sonicated in the buffer containing 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. After centrifugation, the supernatants were passed through a His-Bind nickel-agarose affinity chromatographic column precharged with 50 mM $NiSO_4$ (Novagen, WI). The columns were washed with the washing buffer containing 60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. The proteins were eluted with the elution buffer containing 400 mM imidazole, 500 mM NaCl, and 20 mM Tris-Cl, pH 7.9, and were further purified using a Sephadex G-50 column balanced with phosphate-buffered saline.

Cell Lines and Clones—Jurkat T leukemic cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Jurkat cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, and 100 units/ml each penicillin and streptomycin (complete medium). The generation of stable cell lines expressing epitope-tagged CrmA or Bcl-2 proteins has been described previously (27–30). Transfected cell lines were maintained in complete medium supplemented with 0.5 mg/ml G418 (Life Technologies).

To select for cells resistant to Fas-mediated apoptosis, wild-type Jurkat T cell line was repeatedly exposed to the agonistic anti-Fas mAb, CH-11 (200 ng/ml). Initial treatment induced apoptosis in 99% of the cells. Surviving cells were expanded in culture, and following several consecutive selections with anti-Fas Ab, the cultured cells were further selected by flow cytometry cell sorting to include only Fas-positive cells. Anti-Fas Ab was routinely added to the resistant cell line approximately once a month. Clones of Fas-resistant Jurkat cells were obtained by limiting dilution, and seven of the clonal cell lines were subjected to analysis of mechanisms of resistance. Similar features of resistance to apoptosis were found in all tested clones (as described in this study).

Induction of Apoptosis—Jurkat cells plated at 0.5–1 × 10^6 cells/ml in complete medium were treated with VP-16 (20–40 μM), agonistic anti-Fas Ab (200–500 ng/ml), staurosporin (0.5–1 μM), or TRAIL (100 nM; enhancer, 2 μg/ml) at 37 °C for varying lengths of time, as indicated for each experiment. In several experiments, Jurkat cells were treated with cycloheximide (10 μg/ml) for 4 h prior to the addition of anti-Fas-Ab, CH-11.

Measurements of Apoptosis—DNA fragmentation was assessed by the JAM assay, in which loss of [^{3}H]TdR-labeled DNA was measured (31). DNA labeling of Jurkat target cells was performed by incubation of the cells in the presence of 5 μCi/ml [^{3}H]TdR for 18–24 h at 37 °C. The cells were treated with anti-Fas Ab, VP-16, or staurosporin for various lengths of time, as indicated, and then harvested (Mach IIM, TOMTEC) onto glass fiber filters. The radioactivity of unfragmented DNA, retained on the glass fiber filters, was measured by liquid scintillation counting. Specific DNA fragmentation was calculated according to the following formula: percentage of specific DNA fragmentation = 100 × $(S - E)/S$, where S represents retained DNA in the absence of effector cells (spontaneous) and E represents experimentally retained DNA in the presence of tumor (effector) cells.

Cytofluorometric analyses of apoptosis were performed by costaining with propidium iodide and fluorescein isothiocyanate-annexin V conjugate (CLONTECH). Propidium iodide was used to identify breaks in DNA as a feature of late apoptosis, and annexin V was used to assess aberrant phosphatidylserine exposure (32). The staining was performed according to the manufacturer's directions.

Apoptosis-associated alterations in mitochondria were assessed by flow cytometry analysis using special dyes designed to evaluate mitochondrial events. Disruption of the mitochondrial inner transmembrane potential ($\Delta\Psi m$) was measured by the cationic lipophilic fluorochrome CMXRos. Cells were incubated at 37 °C for 15 min in the presence of CMXRos (0.1 μM, fluorescence at 600 nm) followed by immediate analysis of fluorochrome incorporation (33).

Generation of superoxide anions was measured by HE (Molecular Probes). Cells were incubated at 37 °C for 15 min in the presence of HE (2 μM, fluorescence at 600 nm) followed by immediate analysis of fluorochrome incorporation (34).

Decrease in mitochondrial mass was assessed by NAO (Molecular Probes), reported to measure the inner mitochondrial cardiolipin content (35). Cells were stained with 0.1 μM NAO in RPMI medium for 15 min at 37 °C. Cells were washed and immediately analyzed by flow cytometry.

Staining of cells with anti-APO2.7 served as an additional marker for apoptotic mitochondria. This Ab was raised against apoptotic mitochondria and stains a p38 antigen exposed on apoptotic mitochondria (36).

Preparation of Cytosolic Extracts—Cytosolic extracts were prepared from clones of wild-type or resistant Jurkat cells, as described previously (37). Briefly, cultured Jurkat cells were washed twice with phosphate-buffered saline and then resuspended in ice-cold buffer (20 mM HEPES, pH 7.0, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, 250 mM sucrose, and protease inhibitors). After incubation on ice for 20 min, cells (2.5 × 10^6/0.5 ml) were disrupted by Dounce homogenization (20 strokes). Nuclei were removed by centrifugation at 650 × g for 10 min at 4 °C. Cellular extracts were obtained as the supernatants resulting from centrifugation at 14,000 × g at 4 °C for 30 min.

Caspase-3 activation was initiated by the addition of 10 μM horse

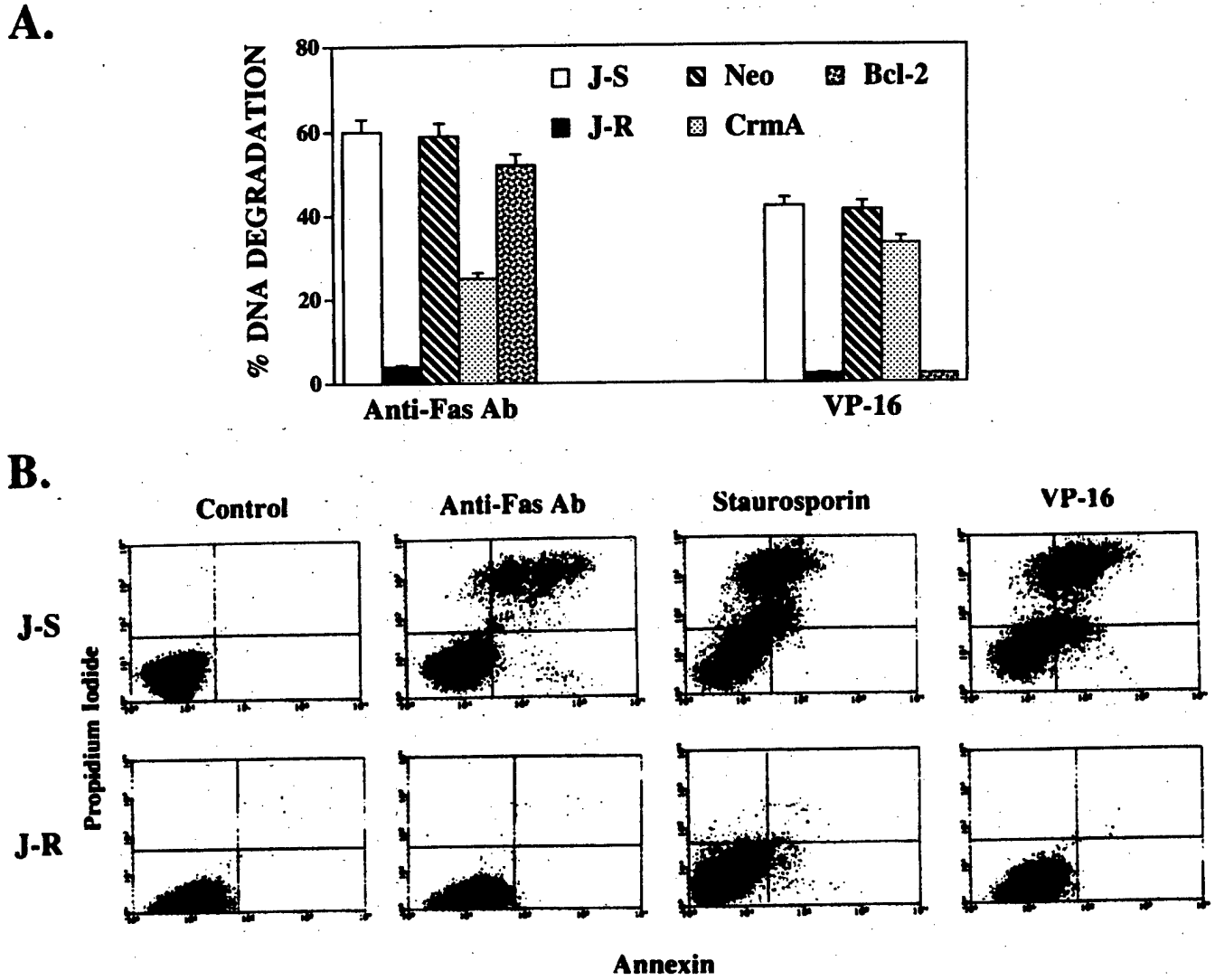

FIG. 1. **Resistance of clonal Jurkat cells to Fas and VP-16 apoptotic signals.** *A*, resistance of Jurkat cells was assessed by the JAM assay as compared with Jurkat cells overexpressing CrmA or Bcl-2. [^{3}H]TdR-labeled clonal Jurkat cell lines were treated with agonistic anti-Fas Ab (CH-11; 200 ng/ml) or VP-16 (20 μM) for 14 h. DNA degradation was measured by loss of [^{3}H]TdR-labeled DNA. The *error bars* represent the S.E. of eight replicates. *B*, resistance to Fas and VP-16 apoptotic signals was assessed by flow cytometry. Clonal Jurkat cell lines were treated with agonistic anti-Fas Ab or VP-16, as described above. The cells were then stained with annexin V (2 μg/ml) and propidium iodide (5 μg/ml), and the presence of apoptotic cells was assessed by flow cytometry.

heart cytochrome *c* and 1 mM dATP to 10 μl of cellular extract at 30 °C for 1 h. To enforce processing of caspase-8, recombinant caspase-3 was added to the cell extracts. The extracts were boiled in sample buffer, resolved by SDS-PAGE, and immunoblotted for processing of caspases.

Western Blot Analysis—To generate whole cell extracts, cells were lysed in 1% Nonidet P-40, 20 mM Tris-base, pH 7.4, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes, as described previously (28, 30). Following probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce).

Subcellular Fractionation—After induction of apoptosis, Jurkat cells were harvested in isotonic mitochondrial buffer (20 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) and Dounce-homogenized by 15–20 strokes (38). Samples were transferred to Eppendorf centrifuge tubes and centrifuged at 650 × *g* for 5 min at 4 °C to eliminate nuclei and unbroken cells. The resulting supernatant was centrifuged at 10,000 × *g* for 30 min at 4 °C to obtain the heavy membrane pellet (HM). The supernatant was further centrifuged at 100,000 × *g* for 1 h at 4 °C to yield the final soluble cytosolic fraction, S-100. HM and S-100 subcellular fractions were assessed for the presence of cytochrome *c* by Western blot analysis.

Mitochondria Purification—Jurkat cells were suspended in mitochondria buffer (MIB) composed of 0.3 M sucrose, 10 mM MOPS, 1 mM EDTA, and 4 mM KH_2PO_4 and lysed by Dounce homogenization as described previously (33). Briefly, nuclei and debris were removed by 10 min of centrifugation at 650 × *g*, and a pellet containing mitochondria was obtained by two successive spins at 10,000 × *g* for 12 min. The washed mitochondria pellet was resuspended in MIB and layered on a Percoll gradient consisting of four layers of 10, 18, 30, and 70% Percoll in MIB. After centrifugation for 35 min at 13,500 × *g*, the purified mitochondria were collected at the 30/70 interface. Mitochondria were diluted in MIB containing 1 mg/ml bovine serum albumin (at least a 10-fold dilution required to remove Percoll). The mitochondrial pellet was obtained by a 30-min spin at 20,000 × *g* and used immediately in the cytochrome *c* release assay.

To assess insertion of Bax or tBid into the mitochondria membrane, purified mitochondria were treated with 0.1 M Na_2CO_3 (pH 11.5) for 20 min on ice to remove alkali-sensitive proteins attached to the membrane (39).

Cytochrome c Release Assay—Purified mitochondria (100 μg of protein) were incubated with recombinant tBid or Bax at various doses as indicated in 20 μl of MIB at 30 °C for 30 min. Mitochondria were pelleted by centrifugation at 4000 × *g* for 5 min. The resulting supernatants or mitochondria were mixed with lysis buffer and analyzed by SDS-PAGE and immunoblotting for the presence of cytochrome *c*.

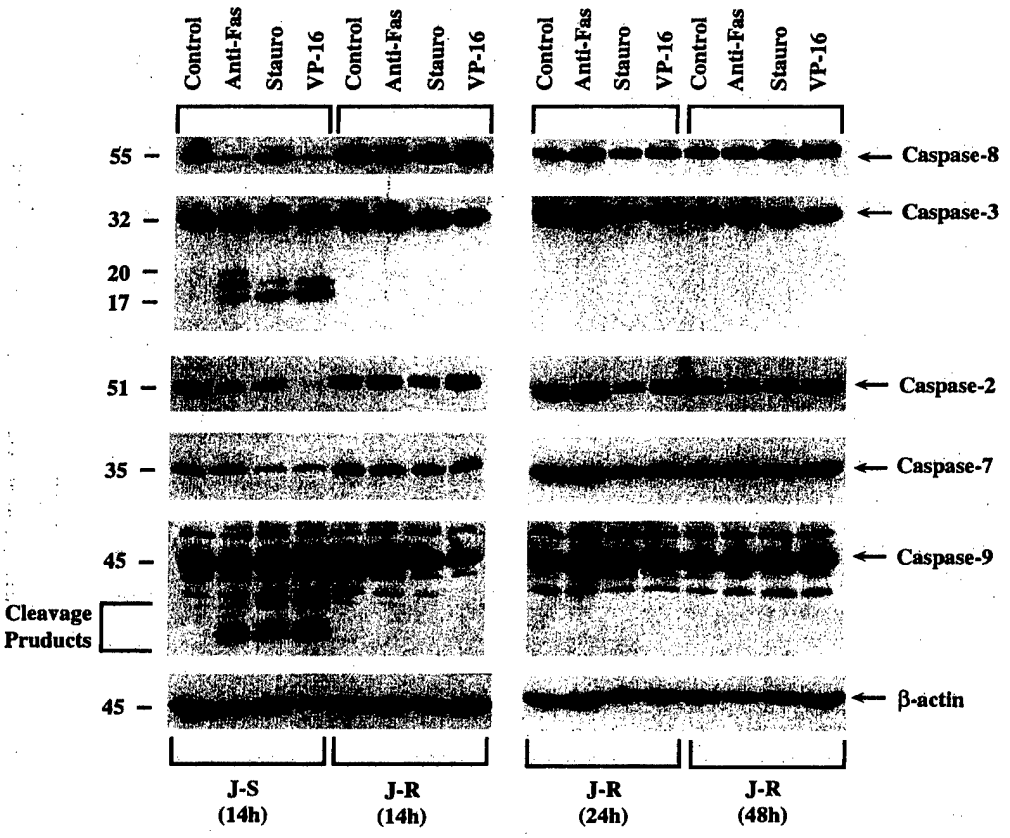

FIG. 2. **Abrogation of caspase processing in resistant clonal Jurkat cells.** Wild-type and resistant Jurkat cells were treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) for 14, 24, or 48 h. At the end of the treatment period, whole cell extracts were separated by SDS-polyacrylamide gels, and resolved proteins were transferred to a polyvinylidene difluoride membrane. The processing of the indicated caspases was assessed by immunoblotting with specific Abs as detailed under "Experimental Procedures." Equal amounts of protein were loaded after quantification, and immunoblotting for β-actin served as an additional loading control. Each *panel* represents the results of at least three experiments.

RESULTS

Cross-resistance to Fas- and VP-16-induced Apoptosis—Selected clonal T cell lines that survived in the presence of agonistic anti-Fas Ab were also found to be completely resistant to apoptotic signals of VP-16 known to initiate a mitochondrial cascade of apoptosis. The degree of resistance to apoptosis induced through the extrinsic or the intrinsic pathway was higher than that observed in Jurkat cells engineered to overexpress either CrmA or Bcl-2. While CrmA-overexpressing Jurkat cells were only partially resistant to Fas-mediated apoptosis, complete resistance was observed in the selected Jurkat cells (Fig. 1*A*). As expected, CrmA-Jurkat cells were highly susceptible to VP-16-induced cell death, whereas the Fas-resistant Jurkat cells were as resistant to VP-16 as Jurkat cells overexpressing Bcl-2 (Fig. 1*A*). These results demonstrate that the mechanism(s) responsible for resistance to apoptosis in the selected Jurkat cells regulate(s) the two known pathways of apoptosis. The cross-resistance to the two pathways of apoptosis was also demonstrated by flow cytometry. As shown in Fig. 1*B*, propidium iodide-positive and annexin-positive cells were detected in Jurkat-sensitive cells treated either with anti-Fas Ab, staurosporin, or VP-16 for 14 h. However, in resistant Jurkat cells treated similarly with either Fas cross-linking or VP-16, no significant level of apoptotic cells was observed. Few apoptotic cells were detected in the resistant clones treated with staurosporin. All clones obtained by limiting dilution of the parental resistant Jurkat cell line possessed cross-resistance to both Fas- and VP-16-apoptotic signals.

Abrogation of Caspase Processing and Activation in Resistant Jurkat Cells—To determine the mechanism involved in resistance to both Fas and VP-16, we tested the processing of endogenous caspases in sensitive and resistant clonal Jurkat cells in response to stimuli by anti-Fas Ab, VP-16, or staurosporin. As shown in Fig. 2, cross-linking of Fas on sensitive Jurkat cells induced processing of caspase-8, -3, -2, -7, and -9. No such processing was observed in resistant Jurkat cells stimulated for periods as long as 14, 24, or 48 h. Likewise, stimulation by VP-16 induced processing of these caspases in sensitive cells but not in the resistant Jurkat cells treated for as long as 48 h. The observed Fas-mediated activation of caspase-9 at 14 h poststimulation of sensitive Jurkat cells results possibly from a mitochondrial amplification loop mediated by caspase-8-cleaved Bid (13–15, 40). Activation of caspase-8 in a late phase of the mitochondrial apoptotic cascade, as seen for VP-16-treated sensitive Jurkat cells, has been previously documented (17). These results demonstrate that caspases known to be involved in either Fas-induced or VP-16-induced cascades are not processed in resistant Jurkat cells. Caspase activation by staurosporin was also significantly blocked in resistant cells as compared with their sensitive counterpart. However, staurosporin-induced processing of caspase-2 was detected in resistant cells following 14 h of treatment. As shown in Fig. 1*B* and corroborated by this analysis of caspase processing, apoptosis induced by staurosporin in resistant Jurkat cells was significantly, but not completely, blocked.

To further assess activity of endogenous caspases, we compared sensitive and resistant Jurkat cells for caspase activity in cleaving known endogenous substrates. The substrates tested included the following endogenous proteins: PARP, known to serve as substrate for caspase-3 and -7 (41); XIAP, demonstrated recently by us (30) and others (42) to be cleaved by caspase-3 and -7; TcR ζ-chain, reported by us to serve as

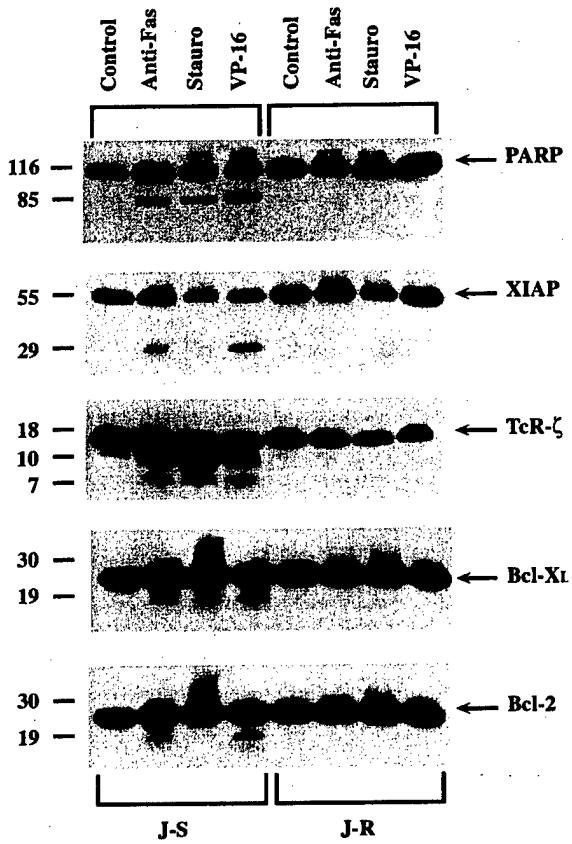

FIG. 3. **Abrogation of caspase cleaving activity of endogenous substrates in resistant clonal Jurkat cells.** Wild-type and resistant Jurkat cells were treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) for 14 h. At the end of the treatment period, whole cell extracts were separated by SDS-polyacrylamide gels, and resolved proteins were transferred to a polyvinylidene difluoride membrane. The cleavage of endogenous substrates was assessed by immunoblotting using specific antibodies to PARP, XIAP, TcR ζ-chain, Bcl-XL, or Bcl-2.

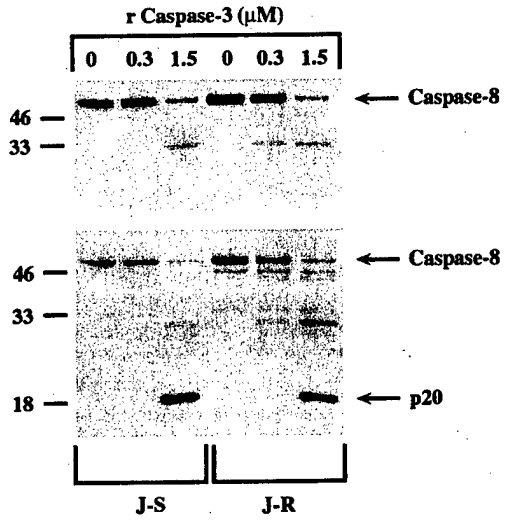

FIG. 4. **Processing of endogenous caspase-8 in extracts of resistant Jurkat cells by recombinant caspase-3.** Recombinant caspase-3 was added to extracts of sensitive or resistant Jurkat cells at 0.3 or 1.5 μM for 30 min at 37 °C. The proteins were resolved by SDS-PAGE and probed with anti-caspase-8 mAb (5F7; UBI) (*top*). The membrane was stripped and reprobed with polyclonal anti-caspase-8 (Ab3; StressGen), which detects the p20 subunit (*bottom*).

substrate for caspase-3 and -7 (28); and the anti-apoptosis proteins Bcl-2 and Bcl-xl, reported to convert to proapoptosis regulators following cleavage by caspase-3 (43, 44). As demonstrated in Fig. 3, no cleavage of any of the substrates tested was detected in resistant Jurkat cells treated with either anti-Fas Ab, VP-16, or staurosporin for 14 h.

Since caspase-8, the apical caspase of the Fas-apoptotic cascade, was not processed in resistant Jurkat clones in response to Fas cross-linking, we examined whether it would be processed by exogenously added caspase-3. Caspase-8 has been shown to be cleaved by caspase-3 downstream of mitochondrial damage induced by γ-radiation (17). As shown in Fig. 4, processing of prodomain caspase-8 as well as the detection of the p20 subunit was similarly observed in extracts of either sensitive or resistant Jurkat cell lines treated with recombinant caspase-3.

Caspase-3 has been identified as an executioner caspase in both the Fas- and VP-16 apoptotic cascades. Therefore, we examined the processing of endogenous caspase-3 in resistant Jurkat cells in the presence of either exogenous recombinant caspase-8 or exogenous cytochrome *c* and dATP. As shown in Fig. 5, caspase-3 subunits were detected in either sensitive or resistant Jurkat cells treated with recombinant caspase-8 or cytochrome *c* and dATP to enforce processing of prodomain caspase-3.

Fas-induced apoptosis in various cells is enhanced in the presence of metabolic inhibitors such as cycloheximide (45). To investigate whether an endogenous protein inhibitor was involved in the observed resistance to the Fas-apoptotic cascade, wild-type and resistant Jurkat cells were treated with cycloheximide prior to the addition of anti-Fas Ab. While an increased percentage of apoptotic cells was detected in sensitive Jurkat cells, this combined treatment did not induce apoptosis in resistant Jurkat cells (data not shown). These observations were further confirmed by assessment of caspase-8 processing. Combined treatment of cycloheximide and anti-Fas Ab did not result in any processing of caspase-8 in resistant cells, while it significantly enhanced the processing seen in wild-type Jurkat cells (data not shown). These findings suggest that the observed block in caspase-8 processing was not mediated by an endogenously metabolized inhibitor.

To further identify the site of blockage, we investigated whether caspase-8 would be processed in response to signals via other death receptors known to use caspase-8 activation as an apical event in their cascade (46). To this end, sensitive and resistant Jurkat cells were cross-linked by TRAIL (100 ng/ml; enhancer, 2 μg/ml) for 14 h at 37 °C. No processing of caspase-8 was detected in resistant cells, whereas similar TRAIL cross-linking induced marked processing in sensitive cells (data not shown). The arrest in caspase-8 activation in both Fas and TRAIL receptor cascades, suggests that the block in the apoptotic cascade in resistant cells is at the level of caspase-8 and not at the receptor level. The block in caspase-8 activation was not mediated by up-regulated expression of FLICE/caspase-8 Inhibitory Protein (FLIP), since similar levels of FLIP-short or FLIP-long proteins were detected in sensitive and resistant Jurkat cells (data not shown). Also, similar levels of FADD expression were detected in sensitive and resistant Jurkat cells (data not shown).

Lack of Apoptosis-associated Alterations in Mitochondria of Resistant Jurkat Cells—To determine the mechanism(s) of the observed resistance of Jurkat cells to VP-16, we assessed apoptosis-associated alterations within mitochondria using fluorescent dyes, which specifically target mitochondrial components or events. Sensitive and resistant Jurkat clones were treated for 8 h with either anti-Fas Ab or VP-16 and assessed for incorporation of the cationic lipophilic dye, CMXRos. Fas cross-linking in sensitive, but not in resistant, Jurkat cells caused significant reduction in incorporation of this dye (Fig.

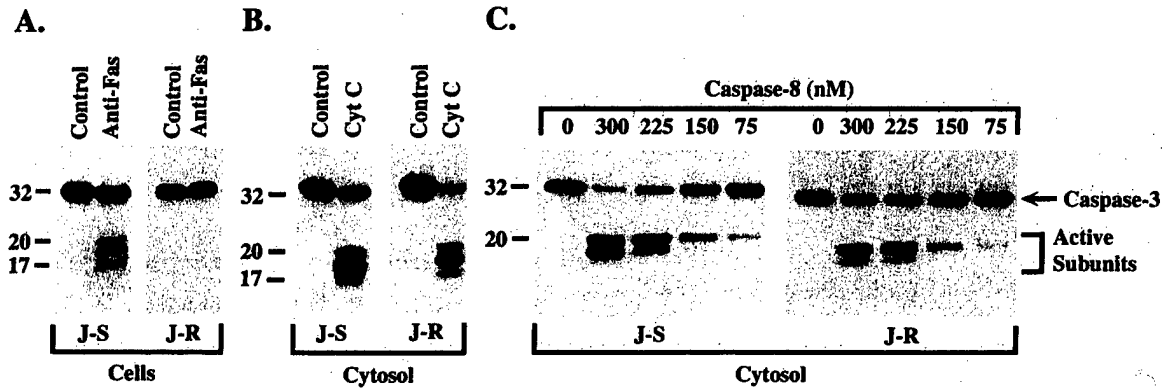

FIG. 5. **Activation of endogenous caspase-3 in extracts of resistant Jurkat cells by exogenous caspase-8 or cytochrome *c*.** *A*, wild-type and resistant Jurkat cells were treated with agonistic anti-Fas Ab (200 ng/ml) for 12 h and immunoblotted by anti-caspase-3 Ab. *B*, cytochrome *c* (10 μg/ml) together with dATP (1 mM; *B*) were added to cytosolic extracts of wild-type or resistant Jurkat cells. *C*, recombinant caspase-8 was added at the indicated doses to extracts of sensitive or resistant Jurkat cells. The extracts (*B* and *C*) were incubated at 30 °C for 1 h and then assessed by immunoblot analysis for the presence of the zymogen and active subunits of caspase-3.

6*A*). These results suggest that alterations in permeability transition in response to VP-16 are blocked in resistant Jurkat cells.

We also assessed mitochondrial changes by NAO, a mitochondria-specific dye, which is independent of permeability transition. NAO is a probe that interacts specifically with nonoxidized cardiolipin, a lipid that is exclusively localized in the inner mitochondrial membrane (35). Loss in cardiolipin, indicating loss in mitochondria matrix integrity, was detected in sensitive cells but not in resistant cells treated with either anti-Fas Ab or VP-16 (Fig. 6*B*). Also, no generation of reactive oxygen species was detected in resistant Jurkat cells, as assessed by flow cytometric analysis of HE staining (Fig. 6*C*).

It has been reported that the mAb APO2.7 detects an apoptosis-associated increase in expression of a p38 mitochondrial antigen (36). A population of cells expressing this antigen was detected in sensitive, but not in resistant, Jurkat cells following Fas or VP-16 stimulation (Fig. 6*D*). These results suggest that the block in the VP-16-induced cascade is at the level of the mitochondria, since mitochondria-specific apoptotic events did not occur in resistant Jurkat cells treated with VP-16.

Involvement of the mitochondria in apoptosis has been associated with release of cytochrome *c* to the cytoplasm, where it initiates a caspase cascade (5, 6). Indeed, in wild-type Jurkat cells treated with either anti-Fas Ab or VP-16, translocation of cytochrome *c* to the cytosol was observed (Fig. 7). However, no cytochrome *c* release was detected in resistant Jurkat cells treated by each of these stimuli. These observations suggest that in resistant Jurkat cells the apoptotic mechanism responsible for cytochrome *c* release is blocked.

Analysis of Intermembrane Protein Release from Purified Mitochondria—It has recently been reported that the recombinant proteins, Bax and tBid, induce cytochrome *c* release when directly applied to purified mitochondria. To further characterize the block in cytochrome *c* release in resistant cells treated with VP-16, we investigated the response of purified mitochondria from sensitive and resistant Jurkat cells to recombinant Bax (GST-BaxΔTM) or tBid (His-tBid). These recombinant proteins have been previously demonstrated to efficiently induce cytochrome *c* release from mitochondria (47–52). Purified mitochondria from sensitive or resistant Jurkat cells were incubated for 30 min at 30 °C with recombinant Bax or tBid. Following removal of mitochondria, the resulting supernatant was tested by immunoblot analysis for the presence of cytochrome *c*. As expected, cytochrome *c* was released from mitochondria obtained from sensitive Jurkat cells in response to either exogenous recombinant Bax or tBid (Fig. 8, *A* and *B*). No release of cytochrome *c* was detected in supernatants of resistant mitochondria treated with these recombinant proteins. These results suggest that the mitochondrial mechanism responsible for release of cytochrome *c* in response to Bid, Bax, or cell stimulation with VP-16 is impaired in mitochondria of resistant Jurkat cells.

To evaluate whether the resistance mechanism was specific for cytochrome *c* or applied also to other intermembrane proteins, we examined release of AIF from sensitive and resistant mitochondria in response to recombinant tBid. As shown in Fig. 8*C*, AIF was released from sensitive, but not resistant, mitochondria. These results suggest that the mitochondrial mechanism responsible for release of intermembrane proteins is blocked in resistant Jurkat cells.

To assess the ability of endogenous Bax or tBid to translocate to the mitochondria, we examined the expression of these proteins within the mitochondria of resistant cells. To this end, purified mitochondria from sensitive and resistant Jurkat cells were treated with 0.1 M Na_2CO_3 (pH 11.5) for 20 min on ice, to remove alkali-sensitive proteins that are attached but not inserted into the mitochondrial outer membrane (39). The presence of Bax within the alkali-resistant fraction of the treated mitochondria was tested for by Western blot analysis. As shown in Fig. 9*A*, similar levels of endogenous Bax were expressed in mitochondria of sensitive or resistant Jurkat cells. The expression of mitochondrial VDAC and the absence of cytosolic β-actin in the alkali-treated mitochondria (*Ins*) further suggest that the fractions tested represent pure mitochondria, and therefore, the translocation of endogenous Bax to the mitochondria is not impaired in resistant cells. These results also demonstrate that in Jurkat cells, as shown previously for HeLa cells (39) and for the hematopoietic cells FL5.12 (53), a basal level of Bax is present within the mitochondria prior to induction of apoptosis. To assess the presence of endogenous tBid in the mitochondria, extracts of sensitive or resistant Jurkat cells were treated with recombinant caspase-8 (100 nM) for 1 h at 37 °C to generate endogenous tBid. The extracts were then separated into cytosol (S-100) and mitochondria (HM). As shown in Fig. 9*B*, endogenous tBid (15 kDa) generated by exogenous recombinant caspase-8 was detected in mitochondrial fractions from both sensitive and resistant Jurkat cells. The use of β-actin as a marker for cytosolic proteins and VDAC as a marker for mitochondrial outer membrane proteins provides evidence for the presence of endogenous tBid in the mitochondria. To further determine whether exogenous tBid added to purified mitochondria could translocate

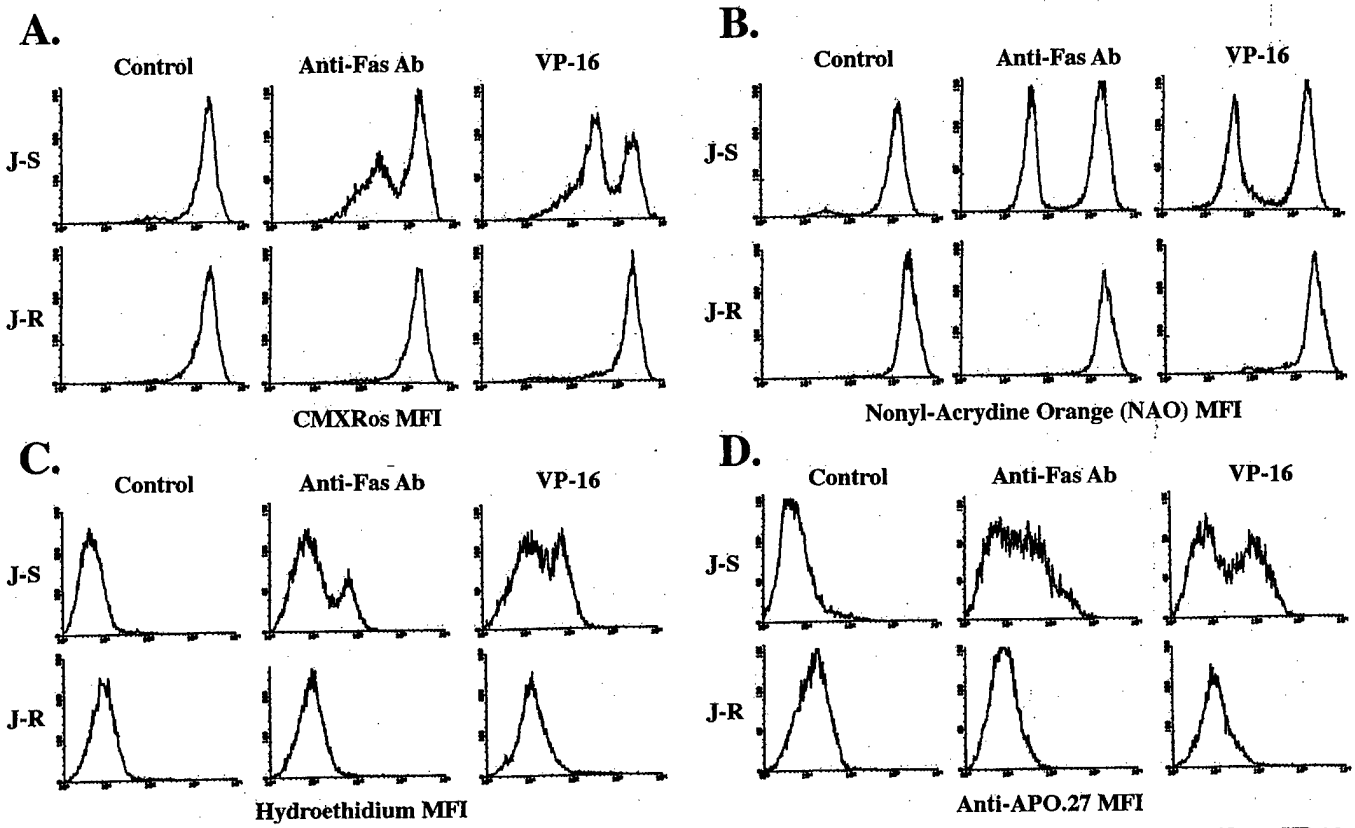

FIG. 6. **Lack of apoptosis-associated alterations in mitochondria of resistant Jurkat cells treated with anti-Fas Ab or VP-16.** Wild-type and resistant Jurkat cells treated with anti-Fas Ab (200 ng/ml) or VP-16 (20 μM) for 8 h were assessed by flow cytometry for mitochondrial changes. Staining with CMXRos (100 nM) served to assess changes in mitochondria permeability transition (*A*); NAO staining (100 nM) served to assess loss in mitochondria cardiolipin (*B*); HE staining (2 μM) served to assess the presence of reactive oxygen species (*C*); and anti-APO.27 Ab (2.5 μg/ml) served to detect a p38 antigen specific for apoptotic mitochondria (*D*).

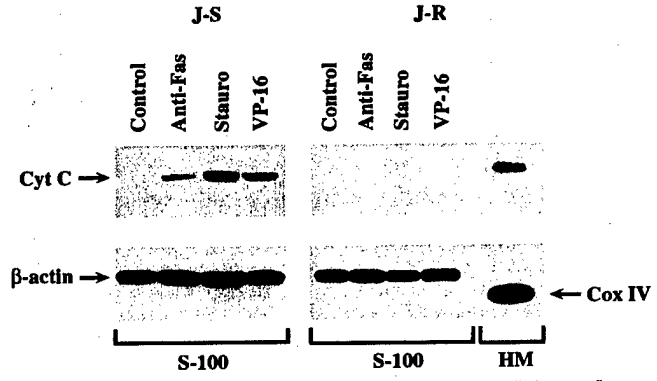

FIG. 7. **Release of cytochrome *c* from mitochondria to the cytoplasm in response to anti-Fas Ab, staurosporin, or VP-16 is blocked in resistant Jurkat cells.** Wild-type and resistant Jurkat cells treated with anti-Fas Ab (200 ng/ml), staurosporin (0.5 μM), or VP-16 (20 μM) for 8 h were assessed for redistribution of cytochrome *c*. Following treatment, Jurkat cells were lysed and separated to an HM fraction, which contains mitochondria, and a mitochondria-free S-100 fraction. The proteins were resolved by 15% SDS-PAGE and immunoblotted by a cytochrome *c*-specific Ab. Equal protein loading was ensured by protein quantification, and by reprobing the stripped membranes with anti-β-actin. Cytochrome *c* oxidase IV served as a marker for mitochondrial fraction. The results shown are representative of at least five independent experiments.

into the mitochondria, purified mitochondria from sensitive or resistant Jurkat cells were incubated with recombinant tBid for 30 min at 37 °C. The mitochondria were then treated with 0.1 M Na_2CO_3, and alkali-sensitive (attached) or alkali-resistant (membrane-inserted) fractions were assessed for the presence of tBid by Western blot analysis. As demonstrated in Fig. 9*C*, tBid was found inserted into the mitochondria membrane in both sensitive and resistant cells. In these studies, cytochrome *c* oxidase IV, a mitochondrial inner membrane enzyme, and VDAC, a mitochondrial outer membrane protein, served as markers for the mitochondrial matrix as well as a gel loading control. These results suggest that the block in cytochrome *c* or AIF release in resistant mitochondria is not mediated by impaired translocation of either Bax or tBid to the mitochondria.

DISCUSSION

In the present study, we describe the selection of Jurkat leukemic T cells resistant to an intrinsic apoptotic pathway via elimination of cells susceptible to the extrinsic Fas-mediated apoptotic cascade. The block in the Fas cascade appears to be at the level of caspase-8, since no processing or activation of caspase-8 was observed following cross-linking of either Fas or TRAIL receptors, which were expressed at a similar level on sensitive and resistant cells. However, caspase-8 in resistant cells was processed in the presence of active recombinant caspase-3, suggesting that it is potentially activable. Activation of caspase-8 was not suppressed by up-regulation in expression of either FLIP-long or FLIP-short or by a differential expression of FADD, since similar levels of these proteins were detected in all sensitive and resistant clones. The resistance to Fas-mediated apoptosis and the block in caspase-8 activation was not affected by pretreatment of the cells with cycloheximide, a metabolism inhibitor that significantly increased susceptibility of sensitive Jurkat cells to Fas-mediated apoptosis. Although these findings suggest that caspase-8 inhibition is not mediated by a metabolized protein inhibitor, the mechanism responsible for the block in caspase-8 activation in these resistant cells is not yet known. It is possible that additional

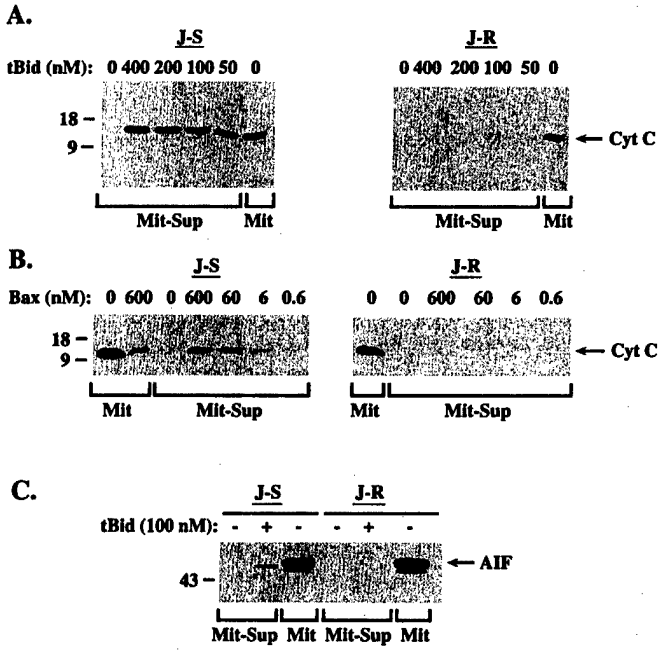

FIG. 8. **Bid and Bax induce release of cytochrome *c* from purified mitochondria of sensitive but not resistant Jurkat cells.** Purified mitochondria from wild-type or resistant Jurkat cells were incubated with the indicated doses of recombinant tBid (*A* and *C*) or recombinant Bax (*B*) for 1 h at 30 °C. Mitochondria were then pelleted by centrifugation, and the resulting supernatant (*Mit-Sup*) was subjected to SDS-PAGE immunoblot analysis with anti-cytochrome *c* Ab (*A* and *B*) and anti-AIF Ab (*C*). Input of an equivalent amount of mitochondria used for each treatment was verified by immunoblot analysis of cytochrome *c* or AIF in control pellets of wild-type and resistant Jurkat cells (*Mit*). The results shown are representative of at least five independent experiments.

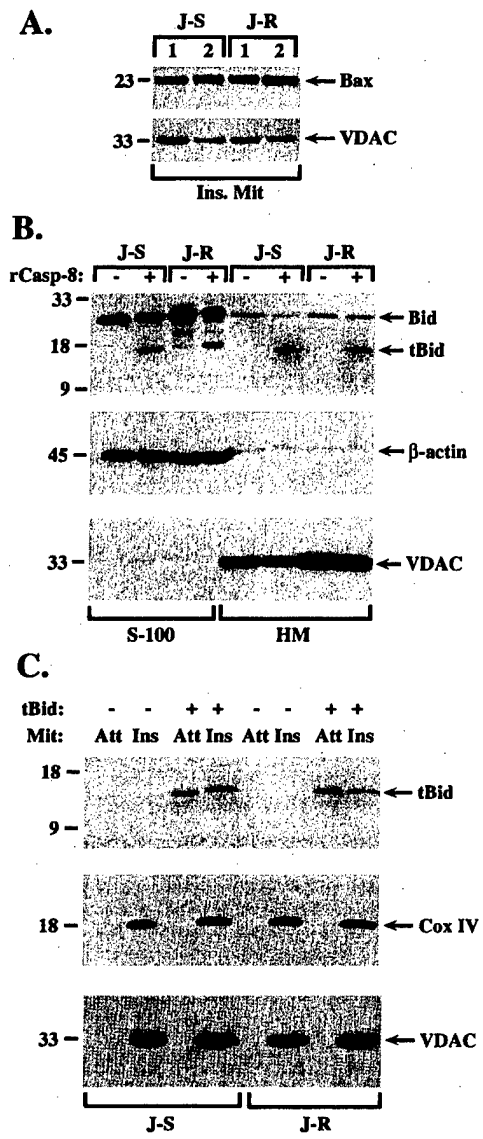

FIG. 9. **Expression of Bax or tBid in mitochondria of resistant Jurkat cells.** *A*, purified mitochondria from sensitive or resistant cells were treated with 0.1 M Na_2CO_3 (pH 11.5) for 20 min on ice. The alkali-resistant mitochondrial fraction (insert (*Ins*)) was lysed, resolved by SDS-PAGE, and immunoblotted for the presence of Bax. Results obtained with mitochondria purified in two different experiments are shown. The blot membrane was stripped and reprobed with anti-VDAC mAb, stripped again, and probed with anti-β-actin. No β-actin was detected in purified mitochondria following alkali treatment (not shown). *B*, extracts of sensitive or resistant Jurkat cells were treated with recombinant caspase-8 (100 nM) for 1 h at 37 °C. The extracts were separated into cytosol (S-100) and mitochondrial (HM) fractions as described under "Experimental Procedures." These two cellular fractions were resolved by SDS-PAGE and probed by immunoblotting for the presence of tBid. The blot membrane was stripped and reprobed with anti-β-actin mAb and subsequently with anti-VDAC mAb, which served as cellular fractionation controls. *C*, purified mitochondria from sensitive or resistant Jurkat cells were incubated with recombinant His-tBid (100 nM) for 1 h at 30 °C. Treated and untreated mitochondria were washed in MIB and further treated with 0.1 M Na_2CO_3 (pH 11.5) for 20 min on ice. Alkali-sensitive (attached (*Att*)) and alkali-resistant (insert (*Ins*)) fractions were lysed, resolved by SDS-PAGE, and immunoblotted for the presence of tBid. The membrane was stripped and reprobed with anti-cytochrome *c* oxidase IV Ab and subsequently with anti-VDAC mAb as markers for mitochondrial matrix and as loading controls.

regulatory components may be involved in the activation of caspase-8, which are yet to be identified. Since caspase-8, the apical caspase in the death receptor cascade, was not activated, it was expected that downstream apoptotic events would not be executed. It was, however, surprising that elimination of Fas-susceptible T cells by anti-Fas Ab would result in selection of cells resistant to intrinsic apoptotic pathways.

The block in the intrinsic cascade was localized to the mitochondria, since no apoptosis-associated alterations were detected in resistant mitochondria treated with VP-16. We tested a broad range of mitochondrial apoptotic events, including changes in the inner membrane, the outer membrane, and the mitochondrial matrix. No changes in inner membrane permeability transition, loss of mitochondrial inner membrane cardiolipin, or reactive oxygen species generation were detected in resistant cell mitochondria. Further, we identified a block in the mechanism responsible for cytochrome *c* release in response to an apoptotic signaling by VP-16 or in cell-free mitochondria treated with recombinant Bax or tBid. The abrogation in release of cytochrome *c* was not due to impaired translocation of either Bax or tBid to the mitochondria. These proapoptotic Bcl-2 family members were found inserted, rather than attached, to the resistant mitochondria. The block in cytochrome *c* release applies also to another intermembrane protein, AIF, reported to be released in response to apoptotic signaling (54, 55). Our results suggest that the block in resistant mitochondria to specific death stimulation localizes to the mechanism responsible for generating specific pores in the outer mitochondrial membrane, which allow translocation of intermembrane proteins to the cytosol.

According to the currently accepted notion, which is based on genetic studies in knockout mouse models (7–10, 12), the initiation phases of the extrinsic and the intrinsic pathways of apoptosis are independent. Cross-talk between the pathways would occur mainly as an amplification loop once the cascade

has been initiated. Fas-induced apoptosis is mediated either by direct activation of downstream effector caspases or via Bid-induced mitochondrial pathway (40). Thus, the observed cross-resistance of Jurkat clones to Fas- and VP-16-mediated apoptosis may be explained as two independent mechanisms involved in blocking the initiation of two independent pathways. Since the entire study was performed in clonal cell lines derived by limiting dilution, each of the clones would encompass two independent mechanisms of resistance. It is possible that in initial selection against cells susceptible to Fas signaling, accumulation of type II cells dependent on mitochondria for their Fas-mediated apoptosis took place (56), whereas continuous selection resulted in cells deficient in mitochondrial mechanism for releasing intermembrane proteins.

The identification of BAR, an apoptosis regulator, which can mediate cross-talk between components of the "independent" pathways, may suggest that in certain cells or apoptotic conditions, common regulators of the two pathways may be functional. BAR was identified by using a yeast-based screen for inhibitors of Bax-induced cell death (57). The BAR protein contains a SAM domain, which is required for its interaction with Bcl-2 and Bcl-XL and for suppression of Bax-induced cell death in both mammalian cells and yeast. In addition, BAR contains a DED-like domain responsible for its interaction with DED-containing procaspases and suppression of Fas-induced apoptosis. Furthermore, BAR can bridge procaspase-8 and Bcl-2 into a protein complex. Since BAR may serve as an inhibitor of Bax and at the same time bind to procaspase-8, we examined by reverse transcription-polymerase chain reaction its level of expression in sensitive and resistant Jurkat cells. We detected similar levels of BAR mRNA in sensitive and resistant Jurkat cells (data not shown), suggesting that BAR is not involved in the mechanism of resistance in the Jurkat cells.

A Fas-interacting serine/threonine kinase capable of inducing phosphorylation of FADD has recently been identified as one of the homeodomain-interacting protein kinases (homeodomain-interacting protein kinase 3) involved in regulation of apoptosis via p53 (58, 59). It is conceivable that such a common regulator of both apoptotic pathways may be linked to the observed mechanism of cross-resistance (58).

A common regulator of Fas- and γ-irradiation-induced apoptotic cascade was also suggested in a previous study (60). Clones of Jurkat cells were reported to have a deficiency in caspase-8 activation in response to Fas cross-linking and in cytochrome *c* release following irradiation. In the present study, we identified the mitochondrial mechanism responsible for the release of intermembrane proteins as the target of the block observed in the intrinsic apoptotic cascade. The biochemical nature of this block and the mechanism responsible for the cross-resistance between the mitochondrial and the death receptor cascades remain to be elucidated.

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A Role for Mitochondrial Bak in Apoptotic Response to Anticancer Drugs*

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In the present study a clonal Jurkat cell line deficient in expression of Bak was used to analyze the role of Bak in cytochrome *c* release from mitochondria. The Bak-deficient T leukemic cells were resistant to apoptosis induced by UV, staurosporin, VP-16, bleomycin, or cisplatin. In contrast to wild type Jurkat cells, these Bak-deficient cells did not respond to UV or treatment with these anticancer drugs by membranous phosphatidylserine exposure, DNA breaks, activation of caspases, or release of mitochondrial cytochrome *c*. The block in the apoptotic cascade was in the mitochondrial mechanism for cytochrome *c* release because purified mitochondria from Bak-deficient cells failed to release cytochrome *c* or apoptosis-inducing factor in response to recombinant Bax or truncated Bid. The resistance of Bak-deficient cells to VP-16 was reversed by transduction of the Bak gene into these cells. Also, the cytochrome *c* releasing capability of the Bak-deficient mitochondria was restored by insertion of recombinant Bak protein into purified mitochondria. Following mitochondrial localization, low dose recombinant Bak restored the mitochondrial release of cytochrome *c* in response to Bax; at increased doses it induced cytochrome *c* release itself. The function of Bak is independent of Bid and Bax because recombinant Bak induced cytochrome *c* release from mitochondria purified from $Bax^{-/-}$, $Bid^{-/-}$, or $Bid^{-/-}$ $Bax^{-/-}$ mice. Together, our findings suggest that Bak plays a key role in the apoptotic machinery of cytochrome *c* release and thus in the chemoresistance of human T leukemic cells.

Mitochondrial function is implicated in the two major apoptotic pathways currently accepted as the model(s) for cell death. The death receptor-mediated pathway involves mitochondria mainly as an amplification loop, whereas cellular stress-mediated apoptosis is regulated predominantly at the level of the mitochondria (1). In both pathways the involvement of the mitochondria is manifested by release of cytochrome *c*, a resident protein in the mitochondrial intermembrane space (2). In the cytoplasm, cytochrome *c* in concert with apoptosis protease-activating factor 1 and dATP activates caspase-9, leading to the subsequent activation of the effector protease, caspase-3 (2, 3). Release of cytochrome *c* is crucial for the mitochondrial pathway of apoptosis, because cells deficient in cytochrome *c* are resistant to UV or cytotoxic drug-mediated apoptosis (4). In addition to cytochrome *c*, other components of apoptosis are sequestered in the mitochondrial intermembrane space, including apoptosis inducing factor (AIF),[1] which when released from the mitochondria induces apoptotic nuclear morphology in a caspase-independent manner (5).

Members of the Bcl-2 family are major regulators of mitochondrial apoptotic events (6). Bcl-2 family proteins can be subdivided into three distinct groups: (i) anti-apoptotic members such as Bcl-2 and Bcl-X_L with sequence homology at BH (Bcl-2 homology)1, BH2, BH3, and BH4 domains; (ii) pro-apoptotic molecules, such as Bax and Bak, with sequence homology at BH1, BH2 and BH3; and (iii) pro-apoptotic proteins that share homology only at the BH3 domain, such as Bid, Bik, Noxa, and Bim (7). Pro- and anti-apoptotic Bcl-2 proteins regulate apoptosis in part by controlling cytochrome *c* release from mitochondria. Expression of Bcl-2 and Bcl-X_L prevents the redistribution of cytochrome *c* in response to multiple death-inducing stimuli (7–9), whereas Bid, Bax, and Bak promote cytochrome *c* release (10–13). These Bcl-2 family members also regulate apoptotic changes in isolated mitochondria; cytochrome *c* release is induced by recombinant Bax, Bak, or Bid and prevented by Bcl-2 or Bcl-X_L (10, 14–17). The ratio between pro- and anti-apoptotic proteins determines in part the susceptibility of cells to a death signal (7, 13, 18).

Bid represents a cytosolic BH3-only protein activated by proteolytic processing. Upon activation of Fas or tumor necrosis factor receptor, Bid is cleaved by caspase-8, and the truncated Bid (tBid) product translocates to the mitochondria and triggers the release of cytochrome *c* (19–21). Immunodepletion of

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[1] The abbreviations used are: AIF, apoptosis-inducing factor; Cox IV, cytochrome *c* oxidase IV; HM, heavy membrane; tBid, truncated Bid; VDAC, voltage-dependent anion channels; NSAID, nonsteroidal anti-inflammatory drug(s); Ab, antibody; mAb, monoclonal antibody; CCCP, carbonyl cyanide *m*-chlorophenylhydrazonel; FITC, fluorescein isothiocyanate; GST, glutathione *S*-transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; MIB, mitochondria buffer; MOPS, 4-morpholinepropanesulfonic acid.

Bid shows that it is required for caspase-8-induced cytochrome *c* release (21). A cleavage product of Bid also induces cytochrome *c* release in response to lysosomal leakage (22) or granzyme B (23, 24). These findings suggest that Bid may serve as a sensor that allows cells to initiate apoptosis in response to the presence of specific activated proteases.

Bax is a p53-regulated protein (25, 26) that participates in the induction of apoptosis in response to a variety of apoptotic signals (26–28). Mutations in Bax have been identified as a mechanism of protection against apoptosis in cancer cells (29–31). A recent study involving Bax-deficient cells has established an unambiguous role for Bax in the response of human carcinomas to anticancer drugs (18). The absence of Bax in a human colorectal cell line completely abolished the apoptotic response to sulindac sulfide or indomethacin, nonsteroidal anti-inflammatory drugs (NSAID).

Bak, a Bcl-2 homologous antagonist/killer has also been reported to be regulated by p53 (32). Bak gene transduction induced cell death and accelerated apoptosis in response to growth factor deprivation in murine lymphoid, neural, and fibroblastic cells (33–35). It has been reported that gastric and colorectal tumors have reduced Bak protein levels compared with normal mucosa (36, 37). Also, mutations of the Bak gene have been identified in human gastrointestinal cancers (31), suggesting that perturbation of Bak-mediated apoptosis may contribute to the pathogenesis of these tumors.

Although interactions between pro- and anti-apoptotic Bcl-2 family members have been extensively studied, interactions among the pro-apoptotic Bcl-2 family members and their potential interdependence has not yet been fully addressed. Cytotoxicity of Bid was reported to correlate with its ability to interact with Bax (38). The interaction of Bid with Bax is associated with a change in the conformation of Bax, its translocation to the mitochondria, and the release of mitochondrial cytochrome *c* (39). However, Bid also induces cytochrome *c* release from Bax-deficient or Bax knockout cells (38, 40), suggesting the involvement of other proapoptotic targets for Bid in mitochondria. Indeed, insertion of tBid into mitochondria from Bax-deficient cells induced a significant up-regulation in the immunoreactivity of mitochondrial Bak to staining by an N terminus-specific anti-Bak Ab (38). The requirement for Bak in tBid-induced cytochrome *c* release has recently been demonstrated in hepatocytes from Bak knockout mice (41). In the current study, utilizing Bak-deficient T leukemic cells, we demonstrate the significance of Bak in the apoptotic machinery of cytochrome *c* release and thus in the chemoresistance of these cells.

EXPERIMENTAL PROCEDURES

Reagents—Staurosporin, etoposide (VP-16), bleomycin, anti-β-actin mAb (clone AC-15), and the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) were purchased from Sigma; cisplatin was from Bristol Laboratories (Princeton, NJ), and sulindac sulfide and indomethacin were from ICN Pharmaceutical (Costa Mesa, CA). A caspase-8-specific mAb (57F) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY); mAbs anti-caspase-7 (clone 51), anti-caspase-2 (clone 47), and rabbit anti-caspase-3 were from Transduction Laboratories (Lexington, KY). An additional rabbit anti-caspase-3 Ab, anti-cytochrome *c* Ab, and Ac-DEVD-AMC caspase substrates were from BD-PharMingen. Rabbit anti-Bid Ab was generated as described previously (42); FITC-annexin V and propidium iodide were from CLONTECH (Palo Alto, CA); anti-human Bak Ab were from Oncogene (Ab-1, mouse clone AMO3, generated against recombinant BakΔC), PharMingen (Ab-2, polyclonal Ab generated against recombinant BakΔC), StressGen (Ab-3, polyclonal Ab generated against residues 2–14 of human Bak), and Santa Cruz (Ab-4, polyclonal Ab generated against peptide within an internal region of human Bak). Anti-mouse Bak Ab was from PharMingen; anti-Bax Ab (N-20) and goat anti- AIF Ab were from Santa Cruz; anti-cytochrome *c* oxidase (Cox) IV was from Molecular Probes (Eugene, OR).

Preparation of GST-Bax, GST-Bak, and His-tagged tBid—Mouse Bax-ΔTM (amino acids 1–173) cDNA was ligated into a pGEX-2T (Amersham Pharmacia Biotech) GST expression vector. The resulting construct encoded a GST-BaxΔTM fusion protein with GST at its N terminus. *Escherichia coli* strain DH5α (Life Technologies, Inc.) cells were transformed and cultured in LB medium. When the A_{600} reached 0.7–1.0, isopropyl β-D-thiogalactoside was added at a concentration of 0.1 mM to induce the expression of the fusion protein. Bacteria were harvested after a 2–3-h incubation at 37 °C and lysed by sonication in lysis buffer (1% Triton X-100, 1 mM EDTA in phosphate-buffered saline). After centrifugation, the supernatant was incubated with preswollen glutathione beads for 30 min at 4 °C. The beads were spun down and washed. GST-BaxΔTM was eluted with 5 mM glutathione in 50 mM Tris-HCl, pH 8.0.

The plasmid pGEX2TKBakΔC encoding C-terminally truncated Bak (missing the last C-terminal 21 residues) as a GST fusion protein was a generous gift from Dr. Thomas Chittenden (Apoptosis Technology Inc. Cambridge, MA). GST-BakΔC was purified after bacterial transformation as described for GST-BaxΔTM.

Mouse tBid (amino acids 60–195) cDNA was cloned into a pET23dwHis vector modified from the parental pET23d(+) vector (Novagen, WI). The expressed tBid has His_6 tag at its N terminus. *E. coli* strain BL21(DE3) cells were transformed and cultured at 37 °C in Terrific Broth. The induction of expression was started at an A_{600} of 0.8–1.0 by the addition of 0.4 mM isopropyl β-D-thiogalactoside with continued incubation of the culture at 37 °C for 2–3 h. The bacterial pellets were resuspended and sonicated in a buffer containing 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. After centrifugation, the cleared supernatants were passed through a His-Bind nickel agarose affinity chromatographic column precharged with 50 mM NiSO4 (Novagen). The columns were washed with wash buffer containing 60 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl, pH 7.9. His-tagged tBid was eluted with elution buffer containing 400 mM imidazole, 500 mM NaCl, and 20 mM Tris-Cl, pH 7.9, and was further purified using a Sephadex G-50 column equilibrated with phosphate-buffered saline.

Cell Lines and Clones—The Jurkat T leukemic cell line was obtained from American Type Culture Collection (Manassas, VA). Jurkat cells were grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, and 100 units/ml each of penicillin and streptomycin (complete medium). A clonal cell line isolated from wild type Jurkat cells was found to be Bak-deficient.

Transduction of Jurkat Cells by Ad/GT-Bak and Ad/GV16—Construction of the Ad/GT-LacZ, Ad/GT-Bak, and Ad/GV16 vectors was reported previously (43, 44). A binary adenoviral vector system was used to overcome Bak-mediated apoptosis in the packaging 293 cell line, as described previously (44). In this binary system, the vector Ad/GT-Bak contains a Bak gene under the control of the GAL4/TATA (GT) promoter and the GAL4/GV16 fusion protein. Bak gene expression could then be induced in target cells by coadministration of the Ad/GT-Bak vector with the second adenoviral vector Ad/GV16, which produces the GAL4/GV16 fusion protein. The transduction efficiencies of the adenoviral vectors were determined by assessing the titer needed to infect the cells with Ad/GT-LacZ and Ad/GV16.

Determination of Caspase Activity—Wild type and clonal Jurkat cells were treated with VP-16 (20 μM), staurosporin (0.5 μM), bleomycin (17 μM), and cisplatin (100 μM) for 4, 12, or 24 h. The cells were Dounce homogenized in extraction buffer (20 mM HEPES, pH 7.2, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, 1 mM EDTA, 10% sucrose, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) on ice. Insoluble proteins were removed by centrifugation. The samples were maintained at −80 °C until assayed. Cytosolic proteins (10–30 μg) were diluted in extraction buffer at a final volume of 200 μl containing 12 μM Ac-DEVD-AMC in a microtiter plate. Release of AMC was detected in a PerkinElmer Life Sciences fluorescence spectrophotometer at an excitation value of 380 nM and emission of 440 nM following 10, 30, or 60 min of incubation at 37 °C.

Western Blot Analysis—To generate whole cell extracts, the cells were lysed in 0.5% Nonidet P-40, 10 mM HEPES, pH 7.4, 142 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. The proteins were resolved by SDS/PAGE and transferred to polyvinylidene difluoride membranes as described previously (45). Following probing with a specific primary Ab and horseradish peroxidase-conjugated secondary Ab, the protein bands were detected by enhanced chemiluminescence (Pierce).

Subcellular Fractionation—To obtain subcellular fractions, including S-100 and HM, Jurkat cell suspension in isotonic mitochondrial buffer (20 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM

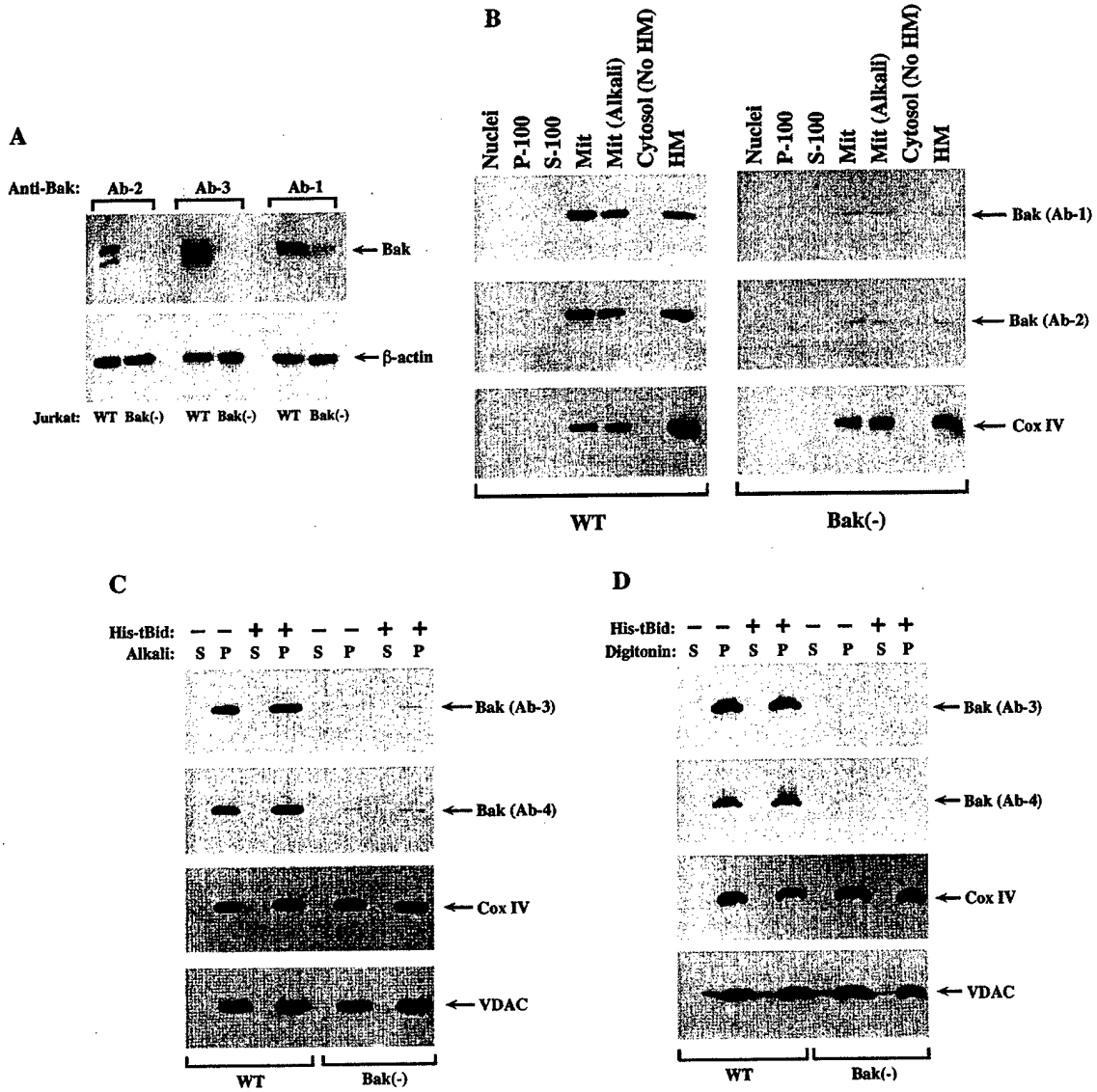

FIG. 1. **Deficient expression of Bak in a clonal Jurkat cell line.** *A*, wild type (*WT*) or clonal Jurkat cells were incubated in 0.5% Nonidet P-40 lysis buffer for 30 min at 4 °C. The resultant lysates, which contained both cytoplasm and mitochondria, were resolved by SDS/PAGE and assessed by immunoblotting for the presence of Bak. Three different anti-human Bak Ab were used for blotting. The membranes were stripped and reprobed for β-actin to demonstrate equal loading. *B*, expression of Bak in mitochondrial fractions of the wild type but not clonal Jurkat cells. Distribution of Bak was examined in subcellular fractions obtained from the two Jurkat cell lines as described under "Experimental Procedures." Subcellular fractions were enriched in nuclei, endoplasmic reticulum (P-100), cytosol (S-100), or HM. Purified mitochondria were treated with alkali to remove nonspecifically attached proteins. The various cell fractions were resolved by SDS/PAGE and immunoblotted for the presence of Bak by Ab-1 (Oncogene). The membrane was stripped and reprobed with anti-Bak Ab-2 (PharMingen). Following additional stripping, the membranes were probed with anti-Cox IV Ab, as a marker for mitochondrial fractions. *C*, expression of Bak within mitochondria is not altered by pretreatment of the mitochondria with His-tBid. Purified mitochondria obtained from the two Jurkat cell lines were treated with His-tBid, followed by exposure to alkali and subsequent separation into alkali-sensitive and alkali-resistant fractions. *S*, supernatant; *P*, pellet. Following SDS/PAGE and electrotransfer, the polyvinylidene difluoride membrane was probed with anti-Bak Ab (Ab-3, StressGen) and then sequentially stripped and reprobed with anti-Bak Ab (Ab-4, Santa Cruz), anti-Cox IV Ab, and anti-VDAC mAb. In *D* purified mitochondria obtained from the two Jurkat cell lines were treated with His-tBid and subsequently separated to digitonin-sensitive (*S*) and digitonin-resistant (*P*) fractions. Following SDS/PAGE and electrotransfer, the polyvinylidene difluoride membrane was successively probed with anti-Bak Ab-3, anti-Bak Ab-4, anti-Cox IV, or anti-VDAC mAb.

$MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin) was Dounce homogenized by 15–20 strokes and centrifuged at 500 × *g* for 5 min at 4 °C to obtain a pellet containing nuclei and remaining intact cells. The postnuclear supernatant was centrifuged at 10,000 × *g* for 30 min at 4 °C to obtain the HM pellet. The supernatant was further centrifuged at 100,000 *g* for 1 h at 4 °C to pellet the remaining membrane (P-100) containing endoplasmic reticulum and to yield the final soluble cytosolic fraction, S-100. HM and S-100 subcellular fractions were assessed for the presence of cytochrome *c* by Western blot analysis. The nuclei, P-100, S-100, and HM were assessed for Bak expression.

Mitochondria Purification—To obtain purified mitochondria, the Jurkat cells were suspended in mitochondria buffer (MIB) composed of 0.3 M sucrose, 10 mM MOPS, 1 mM EDTA, and 4 mM KH_2PO_4, pH 7.4, and lysed by Dounce homogenization as described previously (46). Briefly, nuclei and debris were removed by a 10-min centrifugation at 650 × *g*, and a pellet containing mitochondria was obtained by two successive spins at 10,000 × *g* for 12 min. The washed mitochondrial pellet was resuspended in MIB and layered on a Percoll gradient consisting of four layers of 10, 18, 30 and 70% Percoll in MIB. After centrifugation for 35 min at 13,500 × *g*, the purified mitochondria were collected at the 30/70 interface. Mitochondria were diluted in MIB containing 1 mg/ml bovine serum albumin (at least a 10-fold dilution required to remove Percoll). The mitochondrial pellet was obtained by a 30-min spin at 20,000 × *g* and used immediately.

Alkali or Digitonin Treatment of Purified Mitochondria—Following

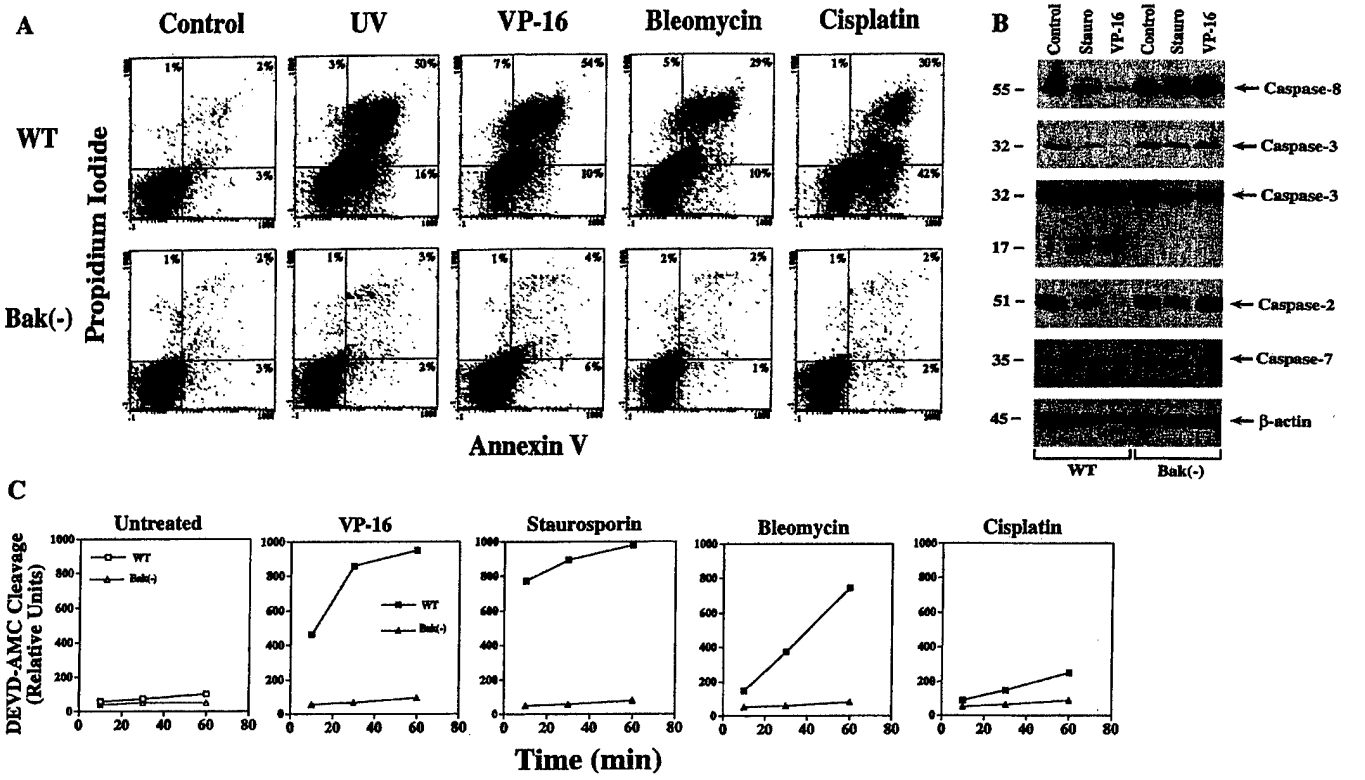

FIG. 2. **Resistance of Bak-deficient Jurkat cells to UV or anticancer drugs.** *A*, flow cytometry analysis of wild type (*WT*) or Bak-deficient (*Bak(−)*) Jurkat cells 24 h after exposure to UV (10 min) or treatment with VP-16 (80 μM), bleomycin (300 μM), or cisplatin (100 μM). The cells were analyzed for staining by FITC-annexin V and propidium iodide. *B*, abrogation of processing of caspases in Bak-deficient Jurkat cells. Wild type or Bak-deficient Jurkat cells were treated with staurosporin (0.5 μM) or VP-16 (20 μM) for 14 h. The cell extracts in 0.5% Nonidet P-40 lysis buffer were assessed by immunoblotting for the expression of the indicated prodomain caspases. Processing of caspase-3 was assessed with polyclonal Ab detecting the prodomain only (Transduction) or prodomain and subunits (PharMingen). Expression of β-actin served as an internal loading control. *C*, abrogation of caspase-3 activity in Bak-deficient Jurkat cells. Wild type or Bak-deficient Jurkat cells were treated for 12 h with VP-16 (20 mM), staurosporin (0.5 μM), bleomycin (100 μM), or cisplatin (100 μM). Caspase activity in cellular extracts (10 μg) was assessed with 12 μM Ac-DEVD-AMC substrate in a final volume of 200 μl of extraction buffer. The results are presented in relative fluorescence units.

incubation of purified mitochondria (100 μg) with recombinant tBid, Bax, or Bak, mitochondria were pelleted and incubated in 100 μl of 0.1 M Na_2CO_3, pH 11.5, for 20 min on ice (47). Alternatively, mitochondria were incubated with 100 μl of digitonin (1.2 mg/ml) for 20 min on ice (48). Supernatants and mitoplasts were separated by centrifugation and dissolved in 0.5% Nonidet P-40 lysis buffer. The fractions were analyzed by immunoblotting.

Cytochrome c Release Assay—Purified mitochondria (100 μg of protein) were incubated with recombinant His-tBid, GST-BaxΔTM, or GST-BakΔC at various doses as indicated in 20 μl of MIB at 30 °C for 30 min. Mitochondria were pelleted by centrifugation at 4,000 × *g* for 5 min. The resulting supernatants or mitochondria were mixed with lysis buffer and analyzed by SDS/PAGE and immunoblotting for the presence of cytochrome *c* or AIF.

RESULTS

Deficient Expression of Bak Protein in Clonal Jurkat Cells—A clonal cell line obtained from the American Type Culture Collection wild type Jurkat cell line was found to be Bak-deficient. The deficiency was determined by immunoblotting of whole cell lysates by three different anti-Bak Abs specific for distinct epitopes (Fig. 1*A*). To further analyze the expression of Bak, lysates of wild type or clonal Jurkat cells were fractionated to yield nuclei, P-100 (endoplasmic reticulum), S-100 (cytosol), purified mitochondria, purified mitochondria treated with alkali to remove loosely attached proteins, cellular extract without HM, or crude HM containing mitochondria. The various protein fractions were assessed for the expression of Bak by Western blot analyses. Whereas expression of Bak was detected in all mitochondria-containing fractions of wild type Jurkat cells, only minor expression of Bak was observed in a similar quantity of purified mitochondria from Bak-deficient cells (Fig. 1*B*). To assess the mitochondrial localization of Bak, purified mitochondria from either wild type or Bak-deficient cells were treated with alkali to remove proteins nonspecifically attached to the mitochondria (47). In wild type Jurkat cells, Bak is a mitochondrial integral membrane protein because it was detected in the pellet of alkali-treated mitochondria (Fig. 1*C*). Because pretreatment of mitochondria with tBid has been reported to enhance Bax localization to the mitochondria (39), we assessed the effects of His-tBid on mitochondrial expression of Bak. However, treatment of mitochondria with His-tBid did not effect Bak localization. In contrast to wild type Jurkat cells, minor or no expression of Bak was detected in mitochondria of Bak-deficient cells, although they express Cox IV or VDAC at levels equal to those of wild type cells (Fig. 1*C*). To further analyze the mitochondrial localization of Bak, wild type or Bak-deficient mitochondria were treated with digitonin (48). Mild digitonin treatment partially lyses the outer membrane but not the inner mitochondrial membrane. In wild type Jurkat cells treated with a low concentration of digitonin, Bak was associated with the pellet of digitonin-treated mitochondria, whereas VDAC, an outer membrane protein, was partially removed from the mitochondria into the digitonin-sensitive fraction (Fig. 1*D*). Partial removal of both Bak and VDAC into the digitonin-sensitive fraction was observed in wild type mitochondria treated with increased digitonin concentrations (data not shown). These findings suggest that in wild type Jurkat cells Bak is a mitochondrial integral membrane protein that is less sensitive than VDAC to removal by mild digitonin treatment. However, the expression of Bak is deficient in the clonal Jurkat cell line.

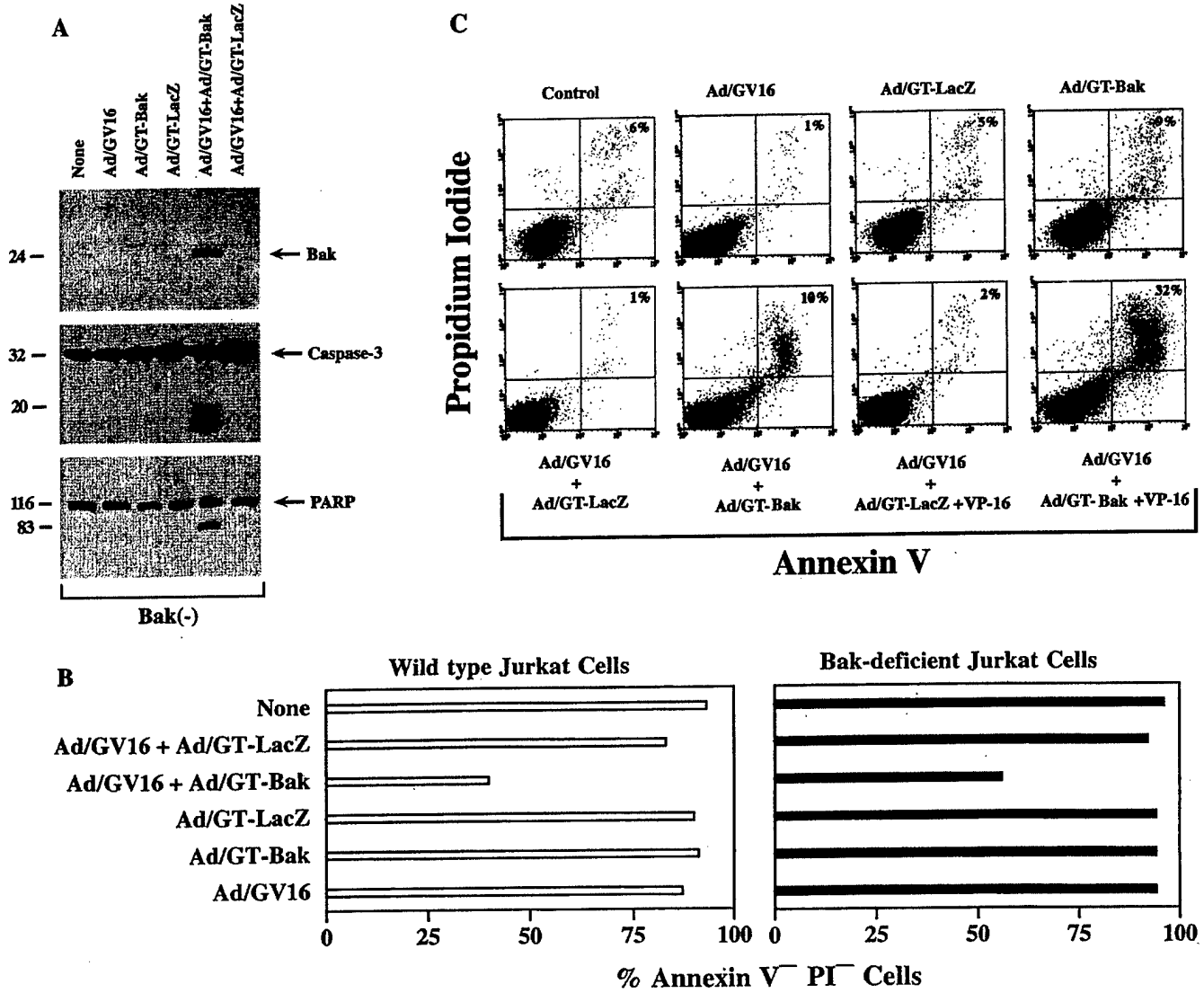

FIG. 3. **Adenoviral-mediated transduction of Bak induces cell death and susceptibility to VP-16 in Bak-deficient Jurkat cells.** *A*, Bak expression following viral infection. Twenty four hours after infection of Bak-deficient cells with the indicated combinations of adenoviral vectors, the cell extracts were assessed by immunoblotting for Bak expression. The membrane was stripped and reprobed successively for caspase-3 activation and poly(ADP-ribose) polymerase (*PARP*) cleavage. *B*, induction of apoptotic cell death within 24 h of infection with the adenoviral binary system for Bak expression in wild type and Bak-deficient cells. Nonapoptotic cells were measured as percentages of annexin V-negative and propidium iodide-negative cells in wild type or Bak-deficient cells infected with the indicated combinations of adenoviral vectors, as assessed by flow cytometry 24 h post infection. *C*, susceptibility of Bak-deficient cells to VP-16 as assessed 48 h post infection. Bak-deficient cells that survived the first 24 h post Bak transduction were assessed for susceptibility to VP-16 (40 μM). Flow cytometry analysis was performed 48 h after infection.

Abrogation of Mitochondrial Apoptotic Pathways in Bak-deficient Jurkat Cell—Because the Bcl-2 proapoptotic family member Bak resides in the mitochondria, we examined the susceptibility of the Bak-deficient cells to apoptotic agents known to initiate mitochondrial pathways of apoptosis. Wild type and Bak-deficient cells were treated with various doses of cytotoxic drugs, including VP-16 (20–80 μM), staurosporin (0.2–1 μM), bleomycin (50–300 μM), or cisplatin (5–100 μM) for 24 h. Whereas wild type cells demonstrated susceptibility to the lowest dose tested for these cytotoxic drugs, the Bak-deficient cells were found resistant even to the highest dose tested for each of the drugs. Following 24 h of treatment with high dose cytotoxic drugs, Bak-deficient Jurkat cells remained negative to staining by FITC-annexin V or propidium iodide, whereas high levels of apoptotic cells were detected in wild type Jurkat cells (Fig. 2*A*). No apoptotic cells were detected among the Bak-deficient cells at 48 or 72 h after exposure to UV or anticancer drugs. No processing of caspase prodomains, as assessed by immunoblot analysis of caspase-8, -3, -2, and -7, was detected in Bak-deficient Jurkat cells treated with staurosporin or VP-16 for 14 h (Fig. 2*B*). Also, no caspase activity as assessed by cleavage of the fluorescent peptide substrate Ac-DEVD-AMC was detected in Bak-deficient Jurkat cells treated with cytotoxic drugs (Fig. 2*C*). These results demonstrate that the Bak-deficient cells are absolutely resistant to apoptotic signals delivered by an array of anticancer drugs.

Transduction of Bak-deficient Cells with Ad/GT-Bak and Ad/GV-16 Vectors—Using a binary adenoviral vector system to avoid the toxic effects of Ad/Bak on 293 packaging cells, we successfully produced large amounts of Ad/GT-Bak, whose gene product (Bak) was under the transcriptional control of the GT promoter and GAL4/GV16 fusion protein (43, 44). The binary adenoviral LacZ vector system (Ad/GT-LacZ and Ad/GV16) was used to determine transduction efficiency. As detected by immunoblotting (Fig. 3*A*), Bak expression was induced when Ad/GT-Bak and Ad/GV16 were administered, but

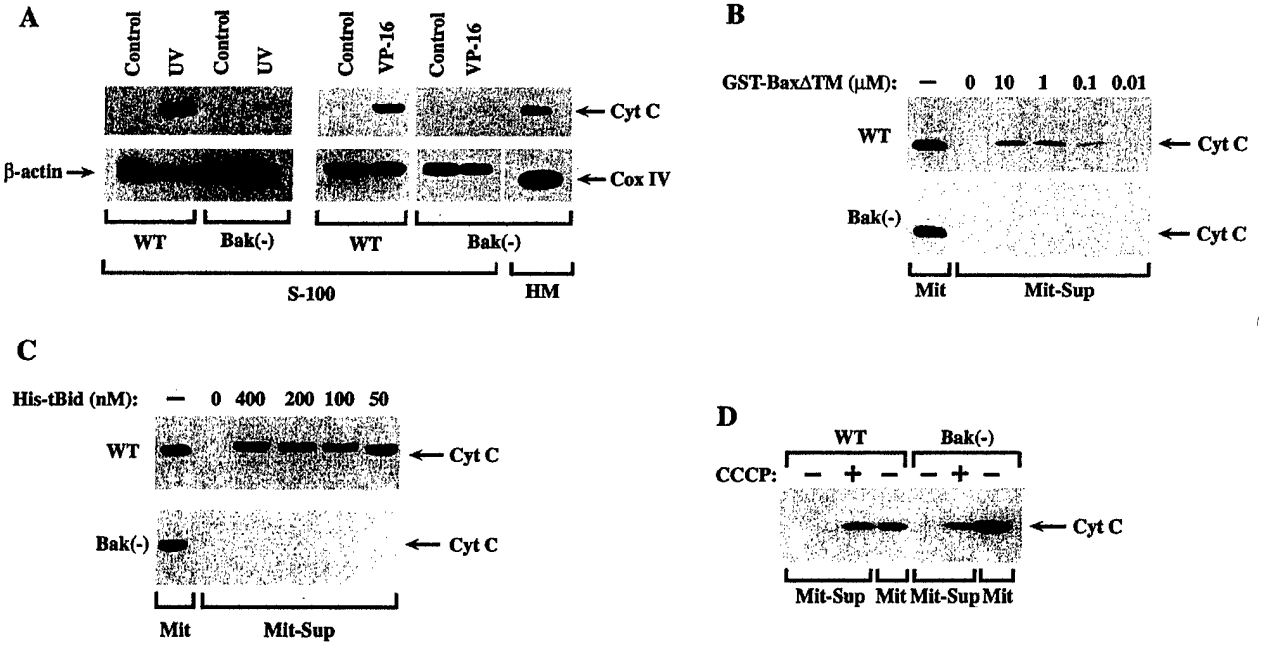

FIG. 4. **Abrogation of cytochrome *c* release in Bak-deficient Jurkat cells.** *A*, wild type (*WT*) or Bak-deficient (*Bak(−)*) Jurkat cells were exposed to UV irradiation for 20 min or treated with VP-16 (20 μM). Twenty four hours later, the cells were Dounce homogenized and separated into a HM fraction, which contained mitochondria and a S-100 cytosolic fraction. S-100 and HM proteins were resolved by SDS/PAGE and immunoblotted with anti-cytochrome *c* Ab. The membranes were stripped, and the S-100 fractions were reprobed with anti-β-actin mAb to demonstrate equal protein loading. HM fractions were reprobed anti-Cox IV Ab as a mitochondrial marker. *B*, no release of cytochrome *c* (*Cyt C*) from Bak-deficient mitochondria in response to recombinant Bax. Purified mitochondria (100 μg) obtained from wild type or Bak-deficient Jurkat cells were treated with GST-BaxΔTM at the indicated concentrations for 30 min at 30 °C. The supernatants (Mit-Sup) were boiled in reducing Laemmeli buffer and assessed by immunoblotting for the presence of cytochrome *c*. Lysed mitochondria from each of the two cell lines tested served as positive controls. *C*, no release of cytochrome *c* from Bak-deficient mitochondria in response to recombinant tBid. Purified mitochondria from wild type or Bak-deficient Jurkat cells were treated with the indicated concentration of His-tBid. Mitochondria from wild type Jurkat cells release cytochrome *c* in response to a dose of His-tBid as low as 5 nM (not shown). *D*, release of cytochrome *c* from wild type or Bak-deficient mitochondria treated with the protonophore CCCP. Purified mitochondria from wild type or Bak-deficient cells were treated with CCCP (50 μM) for 30 min at 30 °C. The supernatants and mitochondria were assessed for the presence of cytochrome *c*.

not when Ad/GT-LacZ and Ad/GV16 were used. A high level of Bak expression has been reported to induce rapid cell death (33). As assessed by flow cytometry of cells stained with propidium iodide and FITC-conjugated annexin V, we also observed a substantial level of apoptotic cell death (40–60%) in both wild type and Bak-deficient Jurkat cells 24 h after transduction with Ad/GT-Bak + Ad/GV16 vectors but not in mock infected cells (Fig. 3*B*). However, at 48 h post infection, when the majority of the initial population of apoptotic cells detected at 24 h has already been disintegrated, a population of cells susceptible to VP-16- or cisplatin-mediated apoptosis was detected in Bak-deficient cells administered with Ad/GT-Bak and Ad/GV16 vectors but not with the LacZ mock vectors (results for VP-16 are shown in Fig. 3*C*). It is plausible that a high level of Bak expression is associated with accelerated cell death, whereas cells with a lower level of Bak expression develop susceptibility to VP-16. Bak-mediated apoptosis was also confirmed by detection of caspase-3 activation and poly(ADP-ribose) polymerase cleavage in Bak-deficient cells infected with Bak but not in mock infected cells (Fig. 3*A*). These results confirm the role of Bak in the observed resistance to VP-16- and cisplatin-mediated apoptosis.

Abrogation of Cytochrome c Release in Bak-deficient Jurkat Cells—Because the mitochondrial apoptotic cascade requires the release of intermitochondrial membrane cytochrome *c* to the cytosol, the Bak-deficient Jurkat cells were assessed for mitochondrial cytochrome c release in response to VP-16 or UV irradiation. In contrast to wild type Jurkat cells, no cytochrome *c* was detected in the cytosolic S-100 fraction of Bak-deficient cells treated with VP-16 or UV irradiation (Fig. 4*A*).

Bid, Bax, and Bak are proapoptotic Bcl-2 family members reported to induce release of cytochrome *c* when applied directly to mitochondria (14, 16, 48). To gain insights into the nature of the block in the cytochrome *c* release mechanism, purified mitochondria obtained from wild type or Bak-deficient Jurkat cells were treated with recombinant Bax (GST-BaxΔTM), and assessed by an immunoblot analysis for the release of cytochrome *c*. GST-BaxΔTM has been shown by mutiple studies to efficiently induce cytochrome *c* release from purified mitochondria (10, 14, 48). Dose-dependent release of cytochrome *c* in response to recombinant Bax was detected in wild type but not in Bak-deficient Jurkat cells (Fig. 4*B*). Likewise, release of cytochrome *c* was detected in supernatants of wild type mitochondria treated with recombinant tBid (His-tBid), whereas the Bak-deficient mitochondria failed to respond to any of the tested doses of recombinant tBid with the release of cytochrome *c* (Fig. 4*C*). However, both wild type and Bak-deficient mitochondria released cytochrome *c* in response to direct exposure to the protonophore, CCCP, which by modulation of membrane potential triggers permeability transition pore opening (Fig. 4*D*) (49, 50).

Because the response to GST-BaxΔTM or His-tBid might be inhibited by increased expression of anti-apoptotic Bcl-2 family members, we examined the expression of Bcl-2 and Bcl-X_L in these cells. Similar levels of expression of Bcl-2 and Bcl-X_L and also of endogenous Bid and Bax were detected in wild type and Bak-deficient cells (Fig. 5, *A* and *B*). Because the lack of response to recombinant Bax or tBid might have been related also to a defect in insertion of these recombinant proteins into Bak-deficient mitochondria, this possibility was next examined. In nonapoptotic cells Bax was reported to reside mainly in the cytoplasm, but a basal level of expression was also present

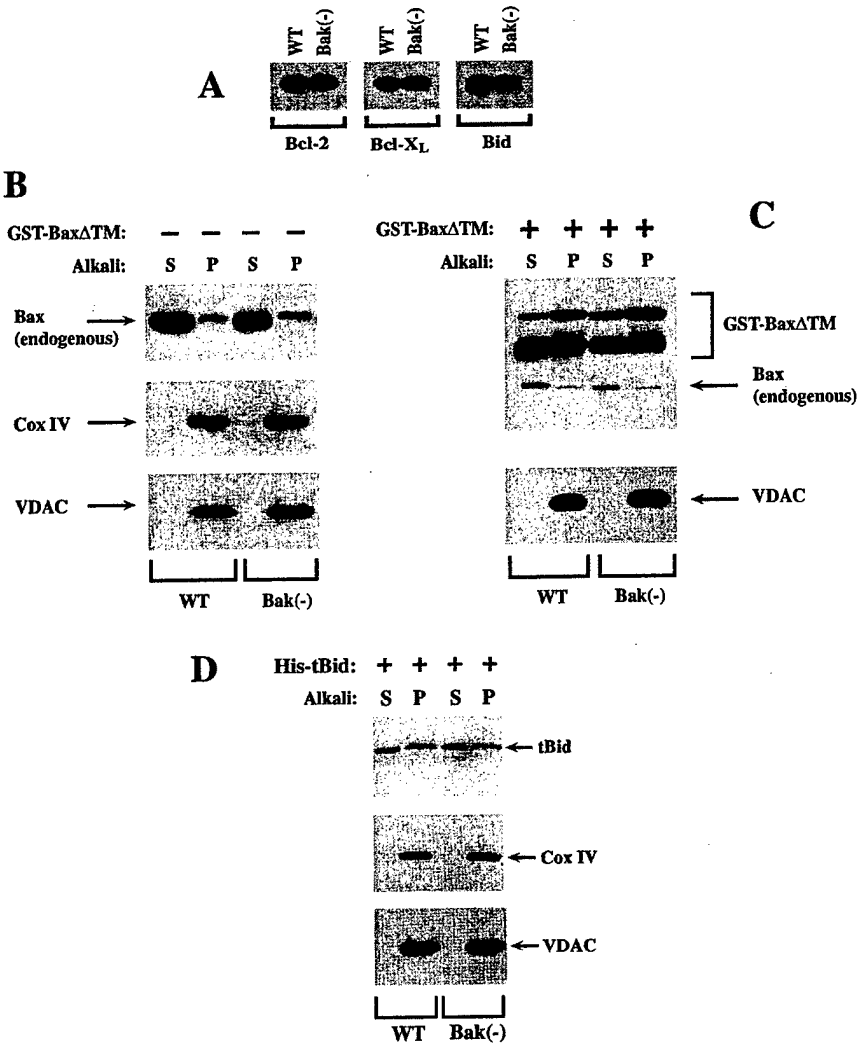

FIG. 5. **Expression of Bcl-2, Bcl-X_L, and Bid in wild type or Bak-deficient mitochondria.** *A*, levels of expression of the indicated proteins were assessed by immunoblot analyses of whole cell lysates. *B*, endogenous Bax is mainly attached to nonapoptotic mitochondria of either wild type or Bak-deficient cells. Purified mitochondria (100 μg) obtained from wild type or Bak-deficient Jurkat cells were treated with 0.1 M Na_2CO_3, pH 11.5, for 20 min on ice. The supernatants (*S*), which contained alkali-sensitive, mitochondria-attached proteins, were separated from the pellets (*P*) of alkali-treated mitochondria, which contained alkali-resistant proteins inserted into the mitochondria. The proteins were resolved by SDS/PAGE and assessed by immunoblotting for the presence of endogenous Bax. The membrane was stripped and successively reprobed with anti-Cox IV Ab and anti-VDAC mAb. *C*, insertion of recombinant Bax into wild type or Bak-deficient mitochondria. Purified mitochondria from wild type or Bak-deficient cells were treated with GST-BaxΔTM. Following alkali treatment, alkali-sensitive and alkali-resistant mitochondrial fractions were assessed for localization of recombinant Bax. The membrane was stripped and immunoblotted for VDAC as a marker for mitochondrial fraction and as a loading control. The appearance of at least two bands of recombinant Bax is likely due to partial degradation of the fusion protein by bacterial proteases. *D*, insertion of His-tBid into wild type or Bak-deficient mitochondria. Purified mitochondria from wild type or Bak-deficient cells were treated with His-tBid, followed by exposure to alkali, as described above. Alkali-sensitive and alkali-resistant mitochondrial fractions were assessed for localization of recombinant tBid. tBid was only detected in mitochondria when exogenously applied (data not shown). The membrane was stripped and successively immunoblotted for the presence of Cox IV and VDAC as markers for mitochondrial fractions and as loading controls. *WT*, wild type.

in the mitochondria (39, 47). Upon induction of apoptosis, Bax translocates to the mitochondria (7, 16, 47, 51). Consistent with these reports, utilizing mitochondria purified from either wild type or Bak-deficient cells, we found that endogenous Bax was attached to the mitochondria, because it was detected in the supernatant of alkali-treated mitochondria. However, a significant portion of the endogenous Bax protein was also inserted into the mitochondrial membrane, because it was also detected in the alkali-treated mitochondrial pellet (Fig. 5*B*). Recombinant Bax incubated with wild type or Bak-deficient mitochondria was also found in both mitochondrial fractions as an attached or inserted protein with no difference between the two cell lines (Fig. 5*C*). We also observed an unaltered insertion of recombinant tBid into Bak-deficient mitochondria (Fig. 5*D*). These observations suggest that the insertion of Bax or tBid into the mitochondria of Bak-deficient cells was unimpaired.

Restoration of Cytochrome c Release from Bak-deficient Mitochondria by Recombinant Bak—As shown in Fig. 3, viral expression of Bak resulted in either a rapid induction of cell death or an acquired susceptibility of Bak-deficient cells to VP-16-mediated apoptosis. We also investigated the possibility of restoring the cytochrome *c* releasing capability of purified Bak-deficient mitochondria following insertion of recombinant Bak. We first examined the ability of recombinant Bak (GST-BakΔC) to insert into the mitochondria of Bak-deficient cells. When applied to purified mitochondria, recombinant Bak had similar patterns of localization in either wild type or Bak-deficient Jurkat cells. GST-BakΔC was detected both as an attached and as an inserted protein in either wild type or Bak-deficient mitochondria (Fig. 6*A*). The insertion of GST-BakΔC withstood mild treatment with digitonin, because it was detected not only in the supernatant but also in the pellet

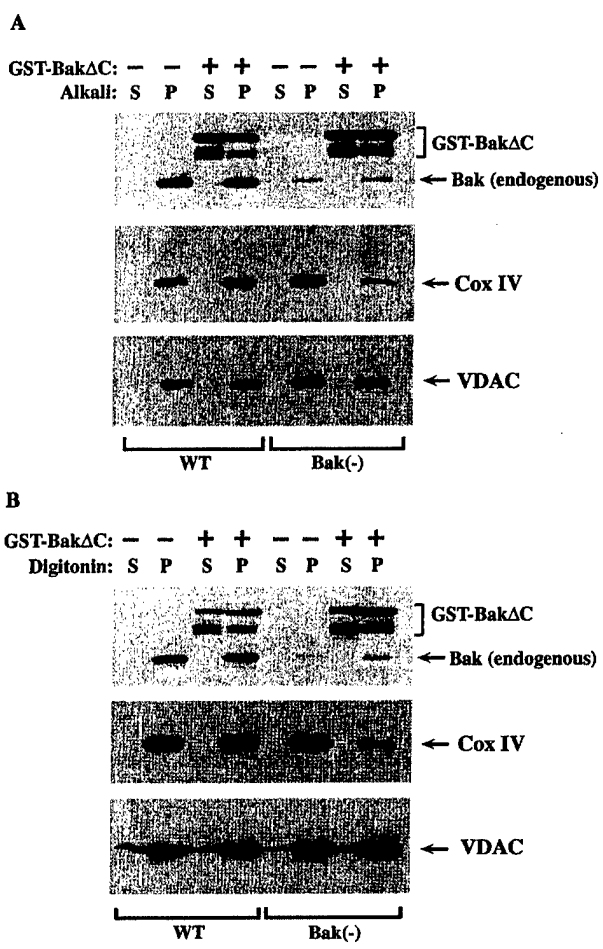

FIG. 6. **Insertion of GST-BakΔC into mitochondria of wild type or Bak-deficient cells.** Purified mitochondria obtained from the two Jurkat cell lines were incubated with GST-BakΔC (8 μM) for 30 min at 30 °C. In *A* the mitochondria were pelleted, washed, and treated with 0.1 M Na_2CO_3, pH 11.5. Alkali-sensitive (*S*) and alkali-resistant (*P*) fractions were resolved by SDS/PAGE and assessed by immunoblotting with anti-Bak (Ab-1, Oncogene) for the presence of endogenous Bak (p24) or recombinant GST-BakΔC. The membrane was stripped and successively reprobed with anti-Cox IV and anti-VDAC mAbs. In *B* the mitochondria were treated with digitonin. The polyvinylidene difluoride membrane was sequentially probed with anti-Bak (Ab-1, Oncogene), anti-Cox IV Ab, and anti-VDAC mAb. The multi-band appearance of GST-BakΔC is likely due to partial degradation of the fusion protein by bacterial proteases. *WT*, wild type.

of digitonin-treated mitochondria (Fig. 6*B*). These observations suggest that recombinant Bak was similarly inserted into wild type and Bak-deficient mitochondria. The multi-band appearance of recombinant Bak was most likely due to degradation of GST-BakΔC by bacterial proteases.

Because recombinant Bak has been reported to induce cytochrome *c* release from liver mitochondria (16), we examined its effect on Bak-deficient mitochondria. Exogenously added recombinant Bak, but not Bax or tBid, induced cytochrome *c* release from Bak-deficient mitochondria (Fig. 7*A*). Recombinant Bak had a dose-dependent effect on cytochrome *c* release in both wild type or Bak-deficient mitochondria (Fig. 7*B*). It also induced the release of another mitochondrial intermembrane protein, AIF, which was detected in the supernatant of Bak-deficient mitochondria (Fig. 7*C*). To investigate the ability of Bak to restore mitochondrial response to recombinant Bax, GST-BakΔC at 0.8 μM, a dose found to be too low to induce cytochrome *c* release, was applied to purified Bak-deficient mitochondria together with GST-BaxΔTM (2 μM). Recombinant Bak was found to synergize with recombinant Bax, because application of combined low doses resulted in release of cytochrome *c* and AIF, which were not released by either Bak or Bax alone at these concentrations (Fig. 7*D*). Thus, the presence of recombinant Bak restored the capability of Bak-deficient mitochondria to release intermembrane proteins in response to recombinant Bax.

Resistance of Bak-deficient Cells to Nonsteroidal Anti-inflammatory Drugs—It has recently been reported that the apoptotic response of colorectal cells to NSAID, including indomethacin and sulindac sulfide, depends on the presence of functional Bax (18). Utilizing Bak-deficient Jurkat cells we investigated a potential role for Bak in resistance to NSAID. Bak-deficient cells were resistant to indomethacin and sulindac sulfide, whereas wild type Jurkat cells were highly susceptible (Fig. 8). These results suggest that in this variant of T leukemic cells the response to NSAID is regulated by Bak.

Bak-mediated Cytochrome c Release Is Independent of Bid or Bax—The expression of Bax and/or tBid in Bak-deficient mitochondria might have enabled cytochrome *c* release mediated by recombinant Bak. To investigate the role of Bax and/or tBid in Bak activity, we examined the effect of GST-BakΔC on cytochrome *c* release from liver mitochondria of either $Bid^{-/-}$, $Bax^{-/-}$, or $Bid^{-/-}$ $Bax^{-/-}$ mice (28, 52). Recombinant Bak induced cytochrome *c* release from wild type liver mitochondria as well as from mitochondria deficient in Bid, Bax, or both Bid and Bax (Fig. 9*A*). These results suggest that mitochondrial Bak is capable of releasing cytochrome *c* independently of Bax or Bid. However, it appears that the release of cytochrome *c* is enhanced when Bak is applied to purified mitochondria in combination with Bax and tBid (Fig. 9*A*). The addition of recombinant Bax or tBid also induced cytochrome *c* release from mitochondria of each of the knockout mice. However, mouse liver mitochondria from wild type, $Bid^{-/-}$, $Bax^{-/-}$, or $Bid^{-/-}$ $Bax^{-/-}$ all expressed similar levels of Bak (Fig. 9*B*).

DISCUSSION

The present study demonstrates a role for Bak in the mechanism of releasing mitochondrial intermembrane proteins by human leukemic cells in response to cytotoxic drugs. The deficiency in Bak endowed Jurkat leukemic cells with a potent mechanism of resistance to UV and drug-mediated apoptosis, which was reversed in viral transduced cells that express Bak. The block in the apoptotic cascade in Bak-deficient cells was localized to the mitochondrial mechanism(s) of releasing intermembrane proteins. No cytochrome *c* was detected in the cytosol of Bak-deficient cells treated with various doses of VP-16, staurosporin, cisplatin, bleomycin, or UV irradiation. Further, in contrast to wild type mitochondria, those isolated from Bak-deficient cells did not release either cytochrome *c* or AIF in response to recombinant Bax or tBid *in vitro*. The deficiency in Bak was directly responsible for the arrest in cytochrome *c* release, because this capability was restored to the Bak-deficient mitochondria by the insertion of recombinant Bak into purified mitochondria from these cells. Furthermore, synergistic activity was detected in the presence of combinations of suboptimal doses of recombinant Bak and Bax, suggesting that in the presence of a low dose of recombinant Bak the resistance of these mitochondria to Bax-mediated cytochrome *c* release was reversed. As recently reported for hepatocytes from $Bak^{-/-}$ mice (41), Bak-deficient Jurkat cells were also resistant to tBid induction of cytochrome *c* release, suggesting that in these leukemic cells Bak is involved in mitochondrial cytochrome *c* release induced by either tBid or Bax.

It has been suggested that tBid acts partly by inducing conformational changes in Bax (38, 39). However, as reported previously (38, 40) and confirmed by this study, recombinant tBid can also induce cytochrome *c* release independently of

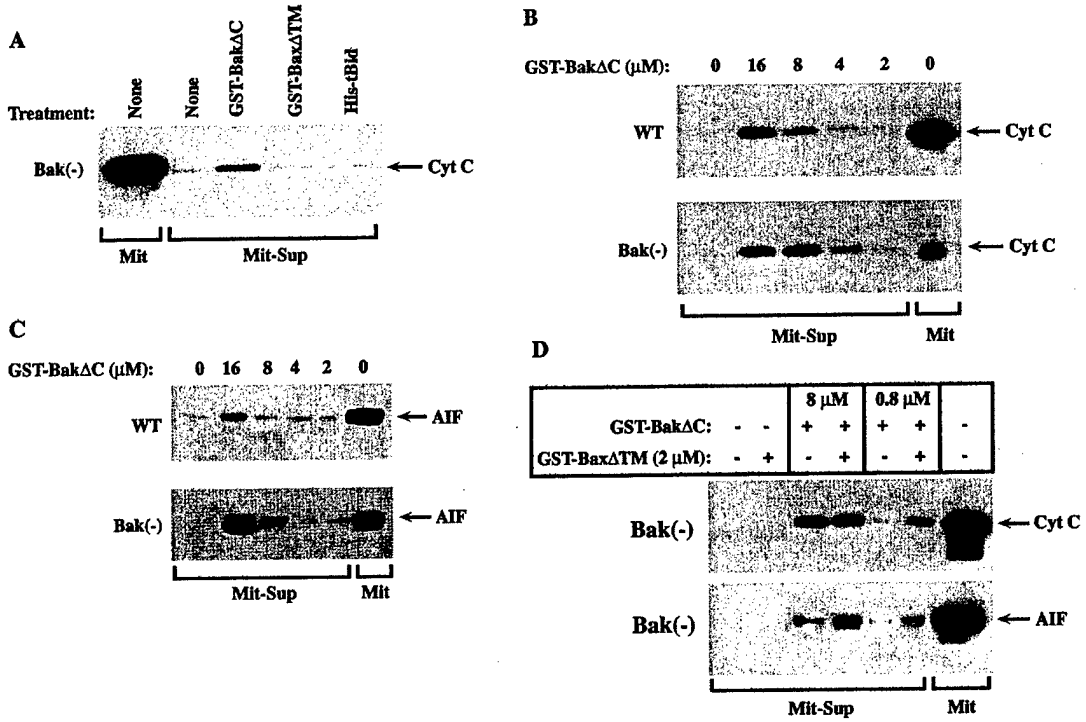

FIG. 7. **Release of intermembrane proteins, cytochrome *c*, and AIF from Bak-deficient mitochondria in response to recombinant Bak.** *A*, Bak-mediated release of cytochrome *c* from Bak-deficient mitochondria. Purified mitochondria obtained from Bak-deficient Jurkat cells (100 μg/20 ml MIB) were incubated with GST-BakΔC (8 μM), GST-BaxΔTM (2 μM), or His-tBid (200 nM) for 30 min at 30 °C. The supernatants were separated from mitochondria, and the proteins of each fraction were resolved by SDS/PAGE and immunoblotted for the presence of cytochrome *c*. *B* and *C*, dose-dependent release of cytochrome *c* (*B*) or AIF (*C*) from wild type or Bak-deficient mitochondria in response to GST-BakΔC. The supernatants of mitochondria treated with various doses of recombinant Bak were assessed for the presence of cytochrome *c* or AIF. *D*, synergism between recombinant Bak and Bax in release of cytochrome *c* or AIF from Bak-deficient mitochondria. Purified mitochondria obtained from Bak-deficient Jurkat cells were treated with recombinant Bak and/or Bax at the indicated doses. The resultant supernatants as well as a sample of mitochondria were assessed by immunoblotting for the presence of cytochrome *c*. The membrane was stripped and reprobed with anti-AIF Ab. *WT*, wild type; *Bak(−)*, Bak-deficient.

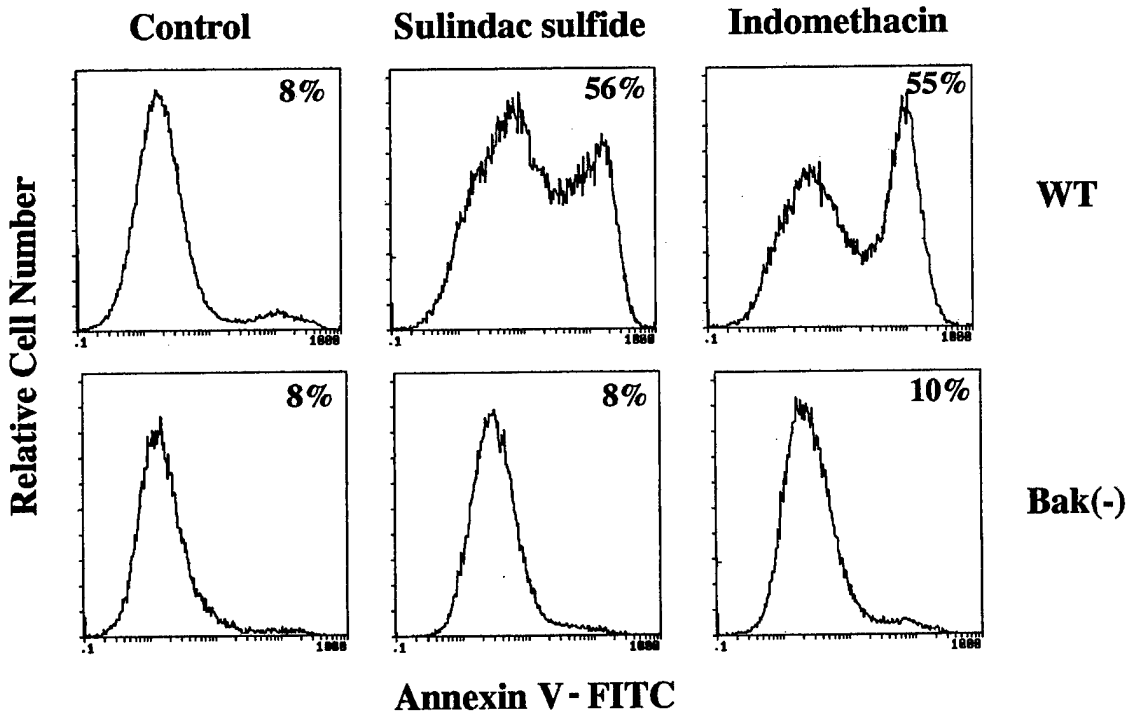

FIG. 8. **Resistance of Bak-deficient cells to sulindac sulfide or indomethacin.** Wild type (*WT*) or Bak-deficient cells (*Bak(−)*) were treated with sulindac sulfide (200 μM) or indomethacin (500 μM) for 36 h. The cells were then stained with propidium iodide and annexin V and assessed for the presence of positive cells by flow cytometry.

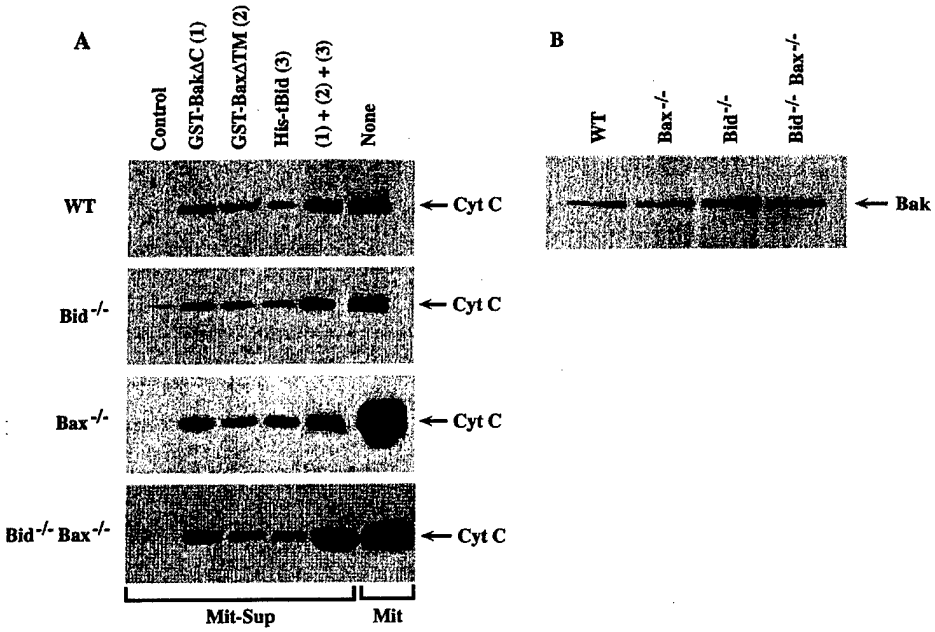

FIG. 9. **Bak-mediated cytochrome *c* release from the mitochondria is independent of Bax and Bid.** *A*, purified mitochondria were obtained from mouse liver of wild type or knockout mice, including $Bid^{-/-}$, $Bax^{-/-}$, and $Bid^{-/-}$ $Bax^{-/-}$. Purified mitochondria (100 μg/20 μl MIB) were treated with GST-BakΔC (4 μM), GST-BaxΔTM (2 μM), or His-tBid (0.2 μM) for 30 min at 30 °C. A combination of recombinant Bak, Bax, and tBid (*(1)+(2)+(3)*) was also assessed. The supernatants of mitochondrial proteins were resolved by SDS/PAGE and assessed by immunoblotting for the presence of cytochrome *c*. *B*, expression of Bak in mitochondria of wild type, $Bid^{-/-}$, $Bax^{-/-}$, or $Bid^{-/-}$ $Bax^{-/-}$ mice. *WT*, wild type; *Bak(−)*, Bak-deficient; *Cyt C*, cytochrome *c*.

Bax. Indeed, Desagher *et al.* (38) have noted that conformational changes in mitochondrial Bak were induced by tBid in mitochondria from Bax-deficient cells. The definitive requirement for Bak in Bid-mediated cytochrome *c* release from mitochondria was recently reported by Wei *et al.* (41). In that study, by treating Bak-expressing mitochondria with anti-Bak blocking Ab or utilizing liver mitochondria from Bak knockout mice, tBid was found to bind in a transient manner to mitochondrial Bak. tBid-Bak binding resulted in oligomerization of Bak as part of a cytochrome *c* release mechanism that was independent of permeability transition (41, 53).

It has recently been reported that $Bak^{-/-}$ mice are developmentally normal and reproductively fit (54). Also $Bax^{-/-}$ mice display limited phenotypic abnormalities (28, 54). However, 90% of mice lacking both Bax and Bak genes died perinatally (54). $Bax^{-/-}$ $Bak^{-/-}$ mice displayed multiple developmental defects, suggesting that Bax and Bak have overlapping roles in regulation of apoptosis during mammalian development (54, 55). These overlapping roles, which were observed in murine models for development of normal cells, remain to be elucidated in human tumor cells. Despite the regulation of Bax and Bak by p53, these tumor suppressors also have pro-apoptotic effects in cells with altered p53 expression (25, 32, 56). Therefore, a redundant function for Bax and Bak, as detected in normal murine cells expressing wild type p53, may be differentially regulated in numerous types of cancer, which are either null or mutated for p53, such as human T leukemic Jurkat cells (57, 58). The study by Vogelstein and co-workers (18) also suggests that the redundancy between Bax and Bak does not apply in human colorectal carcinoma cells, because tumor cells targeted for deficiency in Bax only, but not in Bak, were resistant to NSAID. It is possible that in tumor cells, the balance between Bax and Bak levels of expression is different from that of normal cells. In a case of preferential expression of either Bax or Bak by a tumor cell or in the presence of a nonfunctional form of one of these tumor suppressors, a redundancy in their apoptotic roles may be less apparent. Together, these studies suggest that the roles of Bcl-2 family members in the mechanisms of resistance to anticancer drugs need to be specifically addressed in human cancer cells.

Bak-deficient Jurkat cells and three additional loss-of-function mouse models, including $Bax^{-/-}$, $Bid^{-/-}$, and $Bid^{-/-}$ $Bax^{-/-}$ cells were used in the current study to investigate whether the Bak activation cascade is dependent on Bax or Bid. Recombinant Bak induced cytochrome *c* release from mitochondria of $Bid^{-/-}$, $Bax^{-/-}$, or $Bid^{-/-}$ $Bax^{-/-}$ mice, suggesting that it can function independently of both Bid and Bax. However, we noticed an enhanced cytochrome *c* release from the mitochondria of the various knockout mice examined when treated with a combination of recombinant tBid, Bax, and Bak.

Mounting evidence indicates that apoptosis is the predominant form of cell death triggered by anticancer drugs. Resistance to apoptosis can be acquired by cancer cells through a variety of strategies. In particular, altering a component of the apoptotic machinery has been shown to affect the dynamics of tumor progression, providing a rationale for the inactivation of this machinery during tumor development. Mutations in the Bak gene were detected in human gastric and colorectal cancers (31, 36), and degradation of Bak has been observed in human papillomavirus-positive melanoma (59). However, Bak deficiency as a mechanism for chemoresistance has not yet been addressed. The resistance of Bak-deficient cells to certain anticancer drugs in the face of the susceptibility of wild type Jurkat cells suggests that Bak participates in either the regulation or the execution of the mitochondrial apoptotic response in this type of cancer cells.

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